FERMENTATIONS WITH OXYGEN-ENRICHED AIR

AND

BIOLOGICAL CONTAINMENT OF A CONTINUOUS CENTRIFUGE

A thesis submitted to the Graduate School of the
University of Wisconsin-Madison in partial fulfillment of
the requirements for the degree of Doctor of Philosophy

BY

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Date of Examination

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This thesis is dedicated to my father.

Although military service prevented him from obtaining a college education, he did not need one to give me all that he has given me today.

His one lesson enabled me to obtain the education that he could not. He always gave his total effort to his tasks, never half-hearted.

Had he lived to see the completion of this advanced degree it would have been one of his most proud moments. A difficult task completed.

This is my total effort.

M.C.F.
Blue Mounds, Wisconsin
May, 1977

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This thesis was prepared under the guidance of Professor David Perlman to whom I am sincerely grateful for his patience, direction, and passing on to me a small part of his vast knowledge of the people and processes of the fermentation industry today.

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I would also like to express my appreciation for the support of my wife, Gail. All too often she had to patiently share my attention with billions.

I would also like to acknowledge the financial support of Air Products and Chemicals, Incorporated, and the Research Committee of the graduate school of the University of Wisconsin-Madison who made this program possible.

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INTRODUCTION

The level of dissolved oxygen available for the growth of microorganisms in submerged culture is controlled by four factors: the degree of agitation of the medium, the flow rate of the sparging gas, the partial pressure of oxygen in this gas, and the vessel pressure. During the past forty years, the fermentation industry has relied on varying the agitator speed, aeration rate, or substrate feed to the fermenter in order to control the level of dissolved oxygen. (2,3,4,5,36) This approach has led to the design of industrial fermentation equipment with a large power input for both agitation and aeration in order to obtain sufficient oxygen transfer for high process productivities. Few reports have appeared in the literature concerning manipulation of the oxygen partial pressure (1,6,8,9) or vessel pressure (10).

The basic mathematical relationships describing oxygen transfer in bubble aeration (11,12,13,14,15) (section II) predict that the productivity of a microbial process limited by oxygen transfer can be increased by increasing the partial pressure of oxygen in the sparge gas.

This experimental program is a pilot plant scale study of the application of oxygen-enriched aeration as one possible method for improving the oxygen transfer capability of existing fermentation equipment without increasing the power requirement. This means the use of mixtures of air and compressed oxygen to control the level of dissolved oxygen without significantly increasing the agitation speed, total aeration rate, or vessel pressure. The following areas were investigated for evidence of possible beneficial application of oxygen-enriched aeration to microbial biotransformations or product-forming fermentations:

I. microbial respiration and viability

II. the yield of product formed (without increasing the power requirement of the fermenter)

III. the rate of product formation

IV. utilization of nutrients by the culture

V. regulation of microbial metabolism - control of the products produced or elimination of production of undesirable products.

The following fermentations were studied as test systems: 1) the bioconversion of glycerol to dihydroxyacetone by Gluconobacter melanogenus IFO 3293, 2) the production of the neomycin antibiotics by Streptomyces fradiae 3535, and 3) the production of the bacitracin antibiotics by Bacillis licheniformis 10716. These organisms were grown in 25 liter batch fermentations on complex media under conditions related to those used in industrial production so that the results might be readily applicable to current industrial production practices.

The emphasis of this study is on the physiological response of these organisms when they are grown under conditions of oxygen-enriched aeration. The progress of these fermentations was followed by collecting data on: 1) accumulation of primary product, 2) dissolved oxygen levels, 3) effluent oxygen and effluent carbon dioxide levels, 4) pH, 5) production of cell mass, 6) disappearance of medium carbohydrate, 7) production of other metabolites by the organism, and 8) the ratio of these products and how it is affected by oxygen-enrichment of the aeration.

II. AEROBIC FERMENTATION

A. Mathematical Relationships Describing Oxygen Transfer

Oxygen dissolves in water to an extent which is determined by an equilibrium with an oxygen-containing gas phase. Montgomery (16) has derived an empirical equation expressing oxygen solubility in pure water and saline solution as:

$$C_e = \frac{468}{31.6 + t} \qquad \text{(i)}$$

where t=temperature in °C

C_e = oxygen concentration in mg/l (ppm) in equilibrium with air saturated water vapor at 1 atmosphere pressure

This equation indicates that increasing temperature decreases oxygen concentration. For pure water at 20° C, C_e = 9 mg/liter of dissolved oxygen. The solubility is also decreased by the presence of dissolved salts. Culture media containing dissolved salts and sugars has an oxygen solubility 5 to 20 percent less than that of pure water.

The solubility of oxygen can also be expressed as the partial pressure of gas-phase oxygen in equilibrium with the liquid. This relationship is linear and obeys Henry's law:

$$p = HC_e \qquad \text{(ii)}$$

where H = Henry's law constant for a specific gas composition, liquid phase, and temperature (liter - atm/mg)

p = partial pressure of oxygen in the gas phase (atm)

Using C_e from (i), at 20° C for pure water at 1 atmosphere pressure in equilibrium with air containing 21 percent oxygen, H = 0.023 (liter-atm/mg).

1. Bubble Aeration

For a sparingly soluble gas, such as oxygen, the bulk of the resistance to oxygen transfer is in the transfer across the thin stagnant liquid film that surrounds each air bubble. (17)

With this resistance controlling oxygen transfer, the general relationship governing the rate of oxygen transfer (O.T.R.) in bubble aeration can be described as: (18,19)

$$O.T.R = H\ K_L\ a\ (P_g - P_l) \qquad \text{(iii)}$$

where K_L = oxygen mass-transfer coefficient

a = the interfacial area between liquid and air per unit volume of liquid

P_g = partial pressure of oxygen in the bulk gas phase

P_l = hypothetical partial pressure of oxygen which would be in equilibrium with the actual oxygen concentration in the liquid phase

In the above relationship $(P_g - P_l)$ represents the driving force of oxygen transfer in bubble aeration. Oxygen-enriched aeration increases this driving force by increasing P_g resulting in increased oxygen transfer rates.

2. Physical Factors Affecting Oxygen Transfer

The volumetric oxygen transfer coefficient, $K_L a$, varies during the time-course of a fermentation. (20,21,22) The oxygen transfer coefficient K_L is affected by the viscosity, density, and surface tension of the culture medium (23). These physical characteristics of the broth cannot generally be controlled during the fermentation. For this reason, the majority of the efforts aimed at increasing the oxygen transfer in submerged culture have concerned increasing 'a', the interfacial area between the liquid and air per unit volume of liquid. The value of 'a' is a function of the geometry (design) of the fermentation vessel as shown by the following relationship (24):

$$a \propto \frac{FH_L}{d_B \nu_B V} \qquad \text{(iv)}$$

where F = air flow rate

H_L = liquid depth

d_B = bubble diameter

ν_B = ascending terminal velocity of bubbles

V = liquid volume

This relationship demonstrates that tank diameter, baffling, impeller design, and type of sparger will all effect bubble size and hence, oxygen transfer.

In fermentations with both bubble aeration and mechanical agitation K_L has been shown to be proportional to (impeller speed)$^{\frac{1}{2}}$ (25) and that:

$$K_L a \propto (P_G/V)^{0.4} \nu_s^{0.5} n^{0.5} \qquad \text{(v)}$$

where P_G = power consumption of liquid agitation in a gassed system (Kg m/sec, HP)

V = liquid volume (m^3)

γ_s= superficial air velocity based on cross sectional area of the vessel (m/hr)

n = impeller speec (sec^{-1})

These observed relationships (iv,v) point out that the oxygen transfer rate is directly proportional to the aeration rate and to the (agitation speed)$^{\frac{1}{2}}$. Manipulation of one or both of these factors is commonly used to control the level of dissolved oxygen during a fermentation. These relationships also show that in order to increase the oxygen transfer of a fermenter sparging with air, you must directly increase the amount of power expended in agitation and aeration rate.

3. Oxygen Transfer and Scale-Up in the Fermentation Industry

Scale-up in the fermentation industry is the problem of transfering data from laboratory and pilot plant scale equipment (5 to 3,000 liter) to industrial production (30,000 to 300,000 liters). The accurate determination of the oxygen transfer rate and the amount of power required to maintain this O.T.R. in pilot plant equipment is critical to successful process scale-up (26) The two common methods of scale-up are maintaining 1) constant power per unit volume of liquid or 2) constant volumetric oxygen transfer coefficient (K_La) as the volume of the fermentation is scaled-up. (27) Both the pilot plant equipment and the production equipment have fixed geometry (volume, baffling, impeller speed, etc.) and therefore have different oxygen transfer capabilities. The problems of scale-up concern estimation of the proper impeller speed and aeration rate necessary in the larger vessel in order to duplicate the O.T.R. in the smaller vessel. Oxygen-enriched aeration could offer a new method for obtaining similar oxygen transfer capability in two

fermenters with different geometry.

B. Dissolved Oxygen and Microbial Oxygen Demand

The oxygen uptake rate (O.U.R.) or oxygen demand of a microbial culture is related to the specific growth rate, mass of cells present, and the yield of cells on oxygen:

$$\text{O.U.R.} = \frac{\mu x}{\gamma_{O_2}} \qquad \text{(vi)}$$

where μ = specific growth rate

$$\frac{\text{(gms. cells produced/gms. cells present)}}{\text{hour}}$$

x = gms. cell dry weight present

γ_{O_2} = cell yield on oxygen

$$\frac{\text{gms. cells produced}}{\text{gms. oxygen consumed}}$$

The rate of change of the dissolved oxygen in the fermentation can be described by an oxygen material balance: (28)

rate of change of dissolved oxygen = oxygen transfer rate − microbial oxygen uptake rate

$$\frac{dC}{dt} = K_L a\,(C_g - C_l) - \frac{\mu x}{\gamma_{O_2}} \qquad \text{(vii)}$$

where C = concentration of dissolved oxygen in the liquid

t = time

C_g, C_l = concentration of dissolved oxygen in equilibrium with oxygen partial pressure P_g, P_l respectively.

C_g = O_2 concentration in sparge gas, C_l = dissolved O_2 concentration

As the oxygen demand of the culture begins to approach the oxygen transfer capability of the fermenter, the dissolved oxygen level (C) begins to decrease, eventually to zero. In other words, as cell mass x increases, $(C_g - C_l)$ must increase, hence C_l decreases. If the oxygen demand of the culture is greater than the oxygen transfer capability, the dissolved oxygen level will fall to zero and the culture will be oxygen limited.

1. Measurement of Dissolved Oxygen

Measurement of dissolved oxygen in microbial cultures is normally continuously monitored by means of a membrane probe (29,30,31,32). An alternative method utilizes a submerged Teflon tubing which is continually purged by oxygen-free carrier gas (nitrogen) through a paramagnetic oxygen analyzer (33). The more rapidly responding steam sterilizable galvanic Teflon probes consist of a lead anode, silver cathode, and an acetate buffer as the electrolyte. The current from the electrode is directly proportional to the oxygen activity of the solution not the dissolved oxygen concetration. In order to convert the oxygen electrode reading into concentration, a value for H must be assumed. For most purposes, it is convenient to express the oxygen probe reading in terms of dissolved oxygen tension (DOT), which is in units of atmospheres, or as percent of air saturation.

2. Methods for Control of Dissolved Oxygen Levels in Fermentation

Automatic dissolved oxygen control is necessary in many microbial processes to assure the optimum amount of oxygen for aerobic growth and product formation. All fermentations have a critical lower limit of dissolved oxygen below which respiration and/or product formation becomes

oxygen limited. (12, 34-37)

The most common method for control of dissolved oxygen is by manipulation of agitation speed or aeration rate. (2,5,6,38) Other methods have sought to control dissolved oxygen levels by manipulation of microbial oxygen demand through control of substrate feed (4,7). Several reports have appeared in the literature on control of dissolved oxygen by modification of the driving force of oxygen transfer ($P_g - P_l$). (1,6,8,9) However, these methods increase the P_{O_2} of the sparge gas using mixtures of compressed oxygen and nitrogen. This method of increasing ($P_g - P_I$) may not be economically feasible for large scale application. Oxygen-enriched aeration offers a new method of dissolved oxygen control without manipulation of agitation speed or aeration rate. In addition, oxygen-enrichment of the air can be applied only during the period of maximum oxygen demand while systems which rely on mixtures of oxygen and nitrogen for control must operate throughout the course of the fermentation.

III. MATERIALS & EQUIPMENT

A. Description of the Fermenter

1. Geometry

The fermenter used in these studies is a 50-liter jacketed fermenter (New Brunswick Scientific Company CF-50) operated with a liquid volume of 25 liters. The fermenter was equipped with a 12 cm. diameter ring sparger containing 8 - 4 mm diameter equally spaced holes. Two 10 cm diameter 4 vane disk impellers were spaced 23 cm apart on the impeller shaft. The tank diameter was 31.3 cm and was baffled with 4 baffles of 2.5 cm width each. (figure 1)

Vessel pressure was monitored directly by means of a calibrated stainless steel pressure gauge (0-30 psig Air Products and Chemicals Inc.). Vessel pressure was maintained at 2 - 3 psig throughout all fermentations.

Fermentations were sampled directly by means of a flush mounted drain valve in the bottom of the tank. One hundred ml samples were taken and either refrigerated or immediately frozen and stored until analyzed.

2. Instrumentation

Continuous monitoring of dissolved oxygen tension was made with a galvanic membrane probe (New Brunswick Scientific Company).

In pH controlled fermentations, the pH was maintained at pH 7.0 $\pm$ 0.1 by nonaseptic addition of 4 $\underline{N}$ NaOH by a pH controller (New Brunswick Scientific Company) and two sterilizable pH electrodes (Leeds and Northrup Company).

Figure 2 shows the method of effluent gas analysis. The oxygen concentration in the effluent gas was monitored by means of a

FIGURE 1

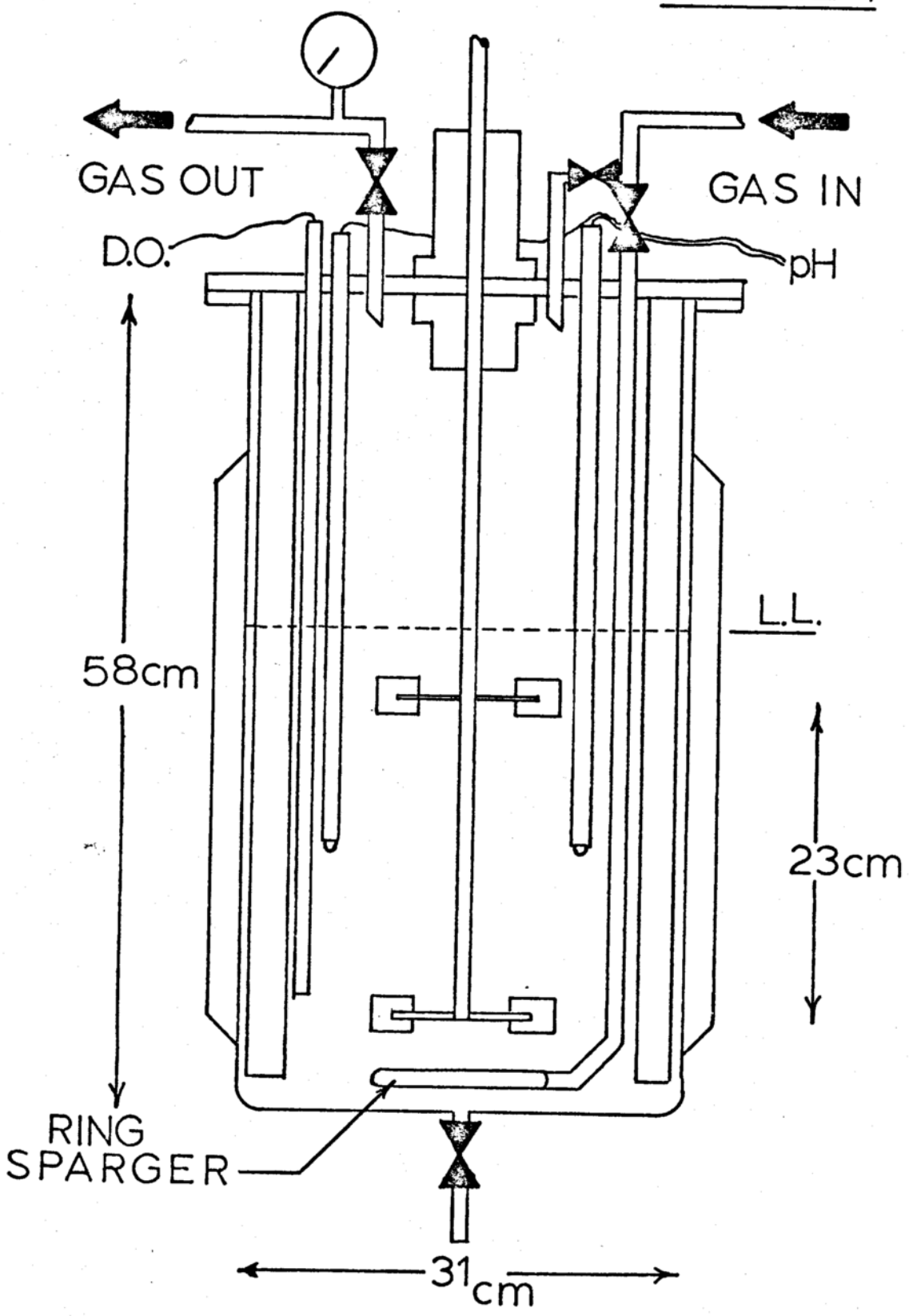
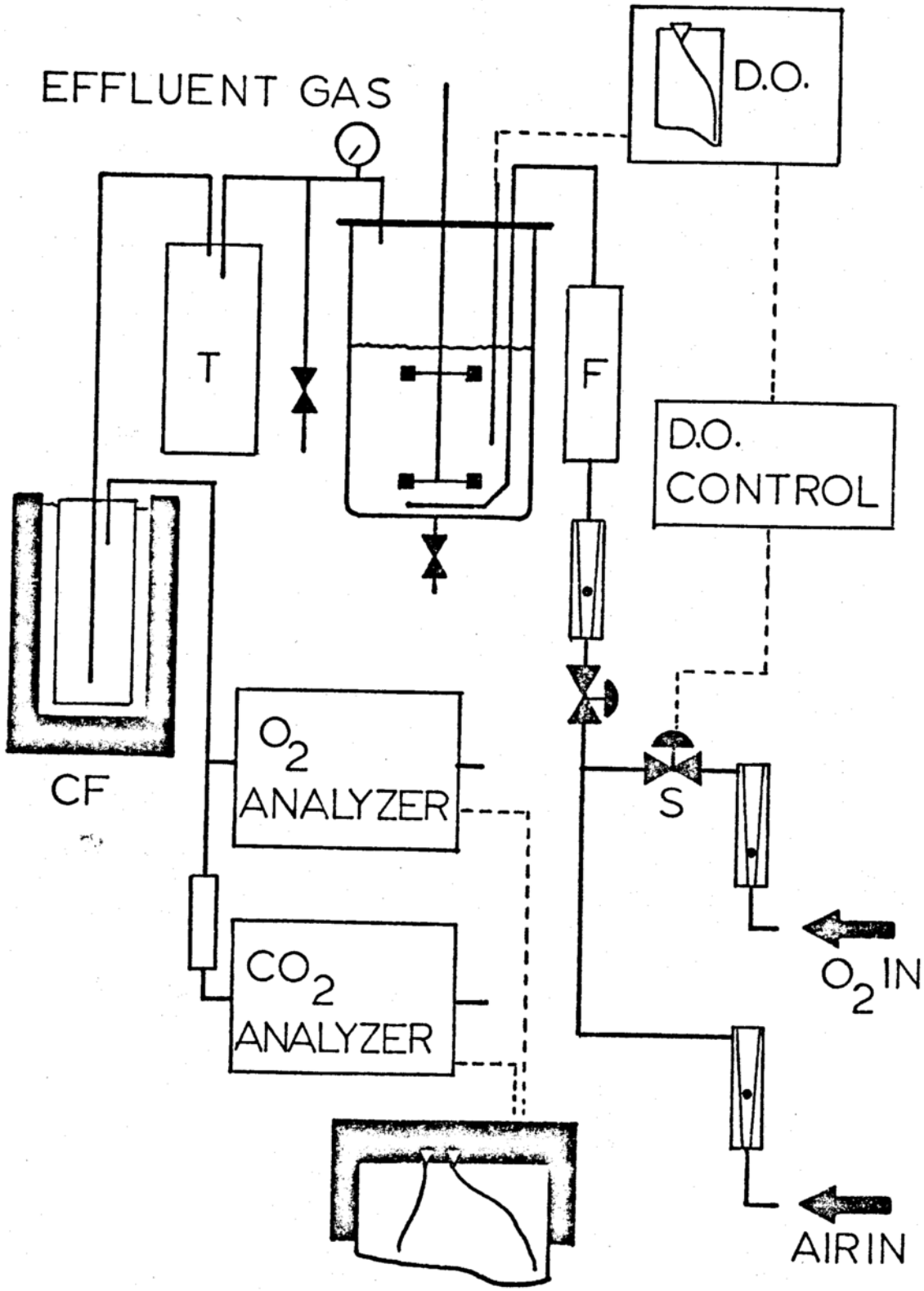


FIGURE 2


EFFLUENT GAS
D.O.
T
F
D.O.
CONTROL
CF
O_2
ANALYZER
S
CO_2
ANALYZER
O_2 IN
AIR IN

paramagnetic oxygen analyzer (Beckman Instruments) calibrated every 12 hours with dry nitrogen and dry air. Effluent carbon dioxide analysis was made using an infra red gas analyzer (Beckman Instruments model 864). Effluent gas from the fermenter first passed through a 5 gallon carboy liquid trap followed by an ice-salt cold finger before entering the oxygen analyzer. Effluent gas for carbon dioxide analysis was further dried by passage through a magnesium perchlorate drying tube. The infra red analyzer was calibrated every 24 hours using dry nitrogen and a standard gas mixture of CO_2 in nitrogen (4.49%, Air Products and Chemicals). All effluent gas lines were clean ¼ inch O.D. copper tubing. (Effluent carbon dioxide analysis was not available for the study of the conversion of glycerol to dihydroxyacetone.)

The output from the pH controller, dissolved oxygen probe, and effluent oxygen and CO_2 analyzers was continuously recorded on strip chart recorders (Rustrack West Instruments, Houston Instruments).

B. Sources of Cultures

The cultures used in this study were obtained from the following sources:

Gluconobacter melanogenus IFO 3293 (Conversion of Glucerol--DHA)	Institute for Fermentation Osaka, Japan
Streptomyces fradiae 3535 W	Waksman Institute for Microbiology, Rutgers University
Bacillus licheniformis ATCC 10716	American Type Culture Collection (ATCC)
Bacillus subtilis (Marburg)	Laboratory culture collection

Bacillus subtilis ATCC 14593	American Type Culture Collection (ATCC)
Micrococcus flavus ATCC 10240-2	American Type Culture Collection (ATCC)

C. Sources of Antibiotics Used as Standard Compounds

Samples of standard antibiotics were obtained from the following sources:

Neamine Neomycin B Neomycin C Bacitracin A + B Bacitracin F Bacitracin X	Dr. G. B. Whitfield, Jr. and Dr. K. Tsuji, Upjohn Company
Bacitracin A lot NE-80171-2	Dr. P. Hidy, IMC Chemical Group Inc.
Neomycin (commercial mixture) lot number 53-NHF-1 675 γ/mg & lot number B-U-697997-6	S. B. Penick & Company
Bacitracin (commercial mixture) lot number 414-NEF-1 50.2 iu/mg	S. B. Penick & Company

IV. METHODS

A. Determination of the Response Time of the Dissolved Oxygen Probe

The response time of the membrane prove was determined at 30°C by the following method:

The probe was immersed in a 3 cm by 24 cm test tube containing approximately 150 ml of distilled water. The test tube was placed in a 30 ± 0.1°C water bath (Precision Scientific Company). Aeration of the distilled water was supplied by an immersed constricted glass tube equipped with a three-way stopcock. Either filtered air or nitrogen gas could be sparged through the vessel.

The 30°C vessel was first vigorously sparged with compressed nitrogen until the dissolved oxygen recorded by the probe was 8-10 percent of air saturation. The stopcock was then quickly switched to sparging with air for 60 seconds introducing oxygen into the water. This increase in dissolved oxygen was followed by a corresponding response by the membrand probe. After 60 seconds, the stopcock was reversed and nitrogen again sparged through the water. The response time of the probe determined by this method was 90 percent of full scale deflection in 60 seconds. This response is sufficiently rapid to give an adequate record of long-term oxygen fluxuations and probes were used without a response correction factor (39).

B. Method Used to Zero the Oxygen Probe

In order to zero the oxygen probe before the start of each fermentation, the hot, sterilized medium was allowed to cool from 121°C to the fermentation temperature without aeration or vigorous agitation. Instead,

sterile air was allowed to enter the fermenter above the medium while the sterile medium cooled by the cooling water of the jacket. In this way, the medium was degassed prior to each fermentation. The reading of the oxygen probe when the medium reached the fermentation temperature and before aeration was started approximated zero dissolved oxygen. One hundred percent of air saturation (0.21 atmospheres dissolved oxygen tension) was determined under conditions of full agitation and aeration before inoculation of the culture.

C. Methods of Addition of Oxygen to the Aeration

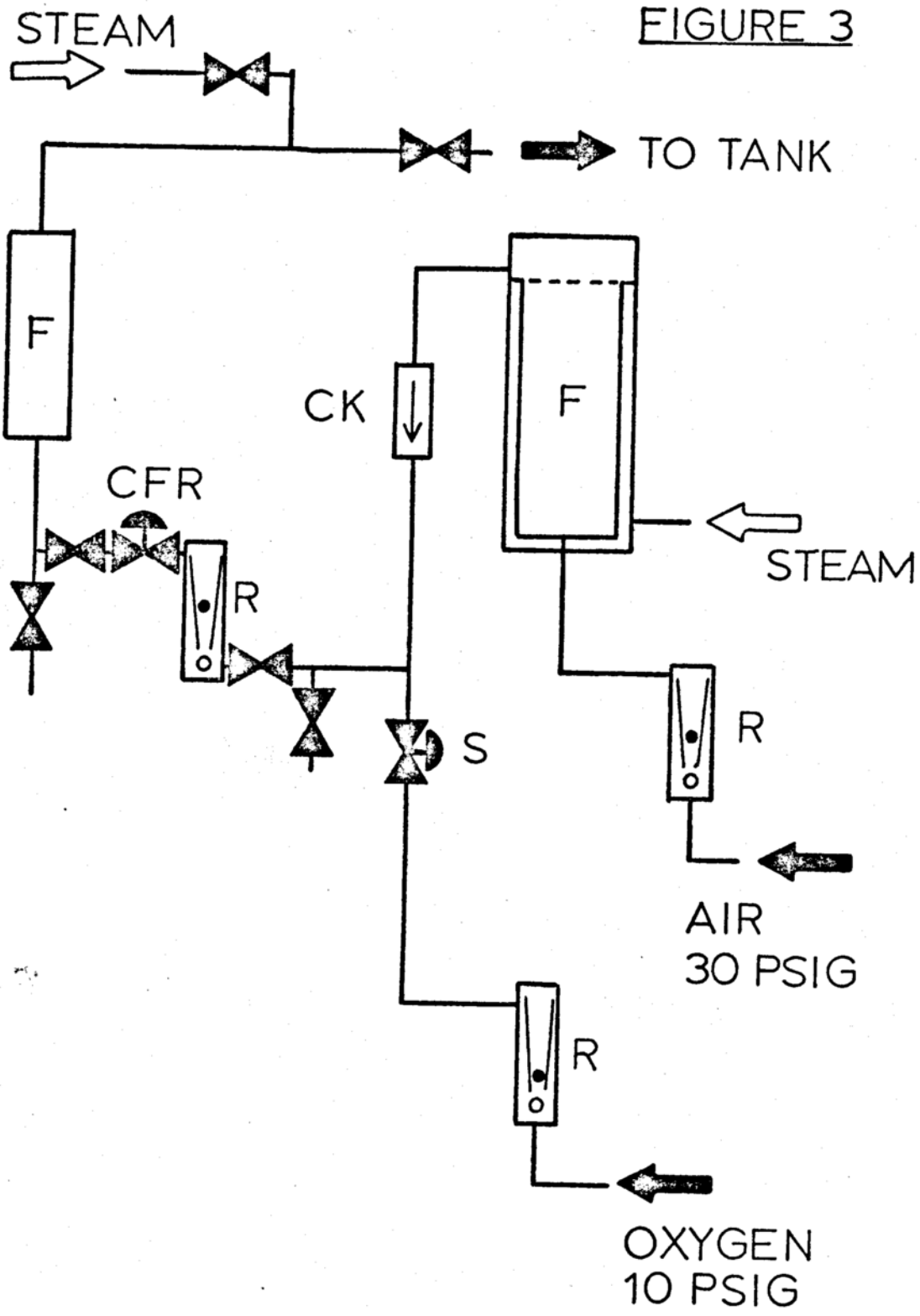
The method of addition of mixtures of air and oxygen is shown in figure 3. The mixture of air and oxygen was sterilized by passage through a steam sterilized glass fiber filter prior to entering the tank. Constant flow rate was maintained by a constant flow regulator (Air Products and Chemicals Inc.). The oxygen was added to the 30 psig air line at a constant pressure of 10 psig. A check valve (600-55-ss Manatrol Co.) was installed up-stream from the point of addition of oxygen to the air line.

Compressed oxygen was metered through high pressure (0-100 psig) and low pressure (0-10 psig) gas regulators (Modern Engineering Co.) equipped with a 0-15 liter per minute flow meter (Modern Engineering Co.). Latex tubing was used to connect the oxygen cylinders to the solenoid valve at the point of addition to the aeration line. The entire aeration line was constructed of 300 series stainless steel.

1. Manual Method of Oxygen Enrichment at Constant Total Aeration Rate

Oxygen enrichment of the fermentation was made in response to physiological oxygen demand of the culture as indicated by the dissolved oxygen probe. Compressed oxygen was manually added to the

FIGURE 3



aeration line beginning at the point in the fermentation where the dissolved oxygen tension reached a level of 0.05 atmospheres. Only enough oxygen was added to maintain this 0.05 atmospheres level. The aeration rate was adjusted so that the total flow rate of the air-oxygen mixtures was the same as when only sparging with air.

As the oxygen demand of the culture decreased, the amount of oxygen was correspondingly decreased so that the 0.05 atm. dissolved oxygen level would not be exceeded. As the oxygen demand further decreased, oxygen enrichment was stopped. (figure 4)

2. Automatic Method of Oxygen Enrichment

An automatic method of oxygen enrichment was devised utilizing a meter relay activating a solenoid valve. (figure 5) The signal from the galvanic oxygen electrode was used as the input for the meter relay. A set point could be adjusted to open the solenoid valve whenever the dissolved oxygen tension fell below the desired control level. In this method of enrichment, oxygen was added to the aeration line in 'shots' at from 5 to 15 liters/minute. The length of the oxygen 'shots' was determined by the oxygen demand of the culture. The total aeration rate using this method of enrichment was not constant but was the sum of the initial aeration rate and the volume of oxygen added. The DOT fluctuations using the automatic method were $\pm$ 0.005 atm. while with manual control $\pm$ 0.015 atm.

D. Maintenance of Cultures

Gluconobacter melanogenus IFO 3293 was maintained on agar slants consisting of: L-sorbose 10 gm/l., yeast extract (Amber 1003) 1 gm/l.,

FIGURE 4

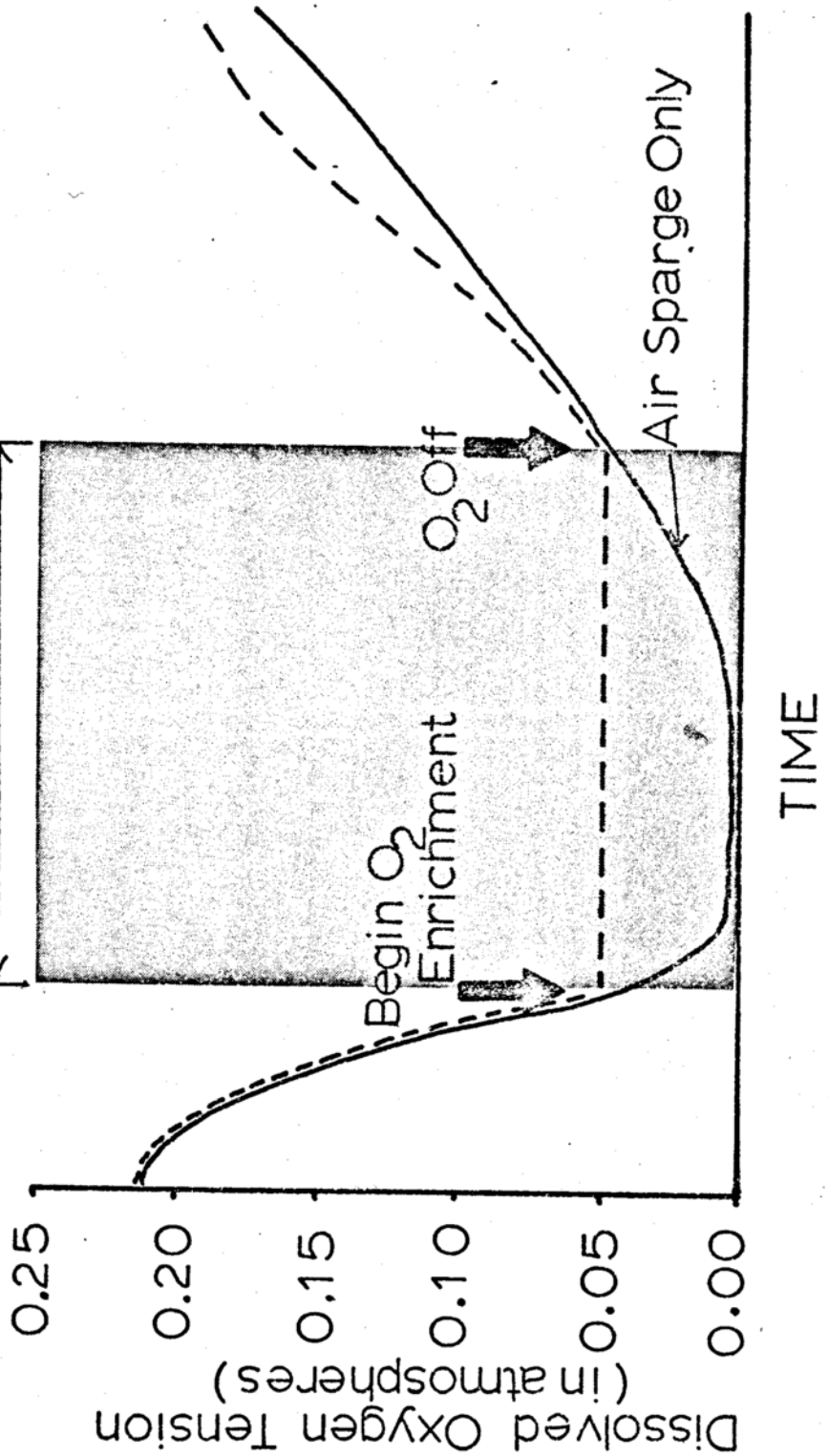
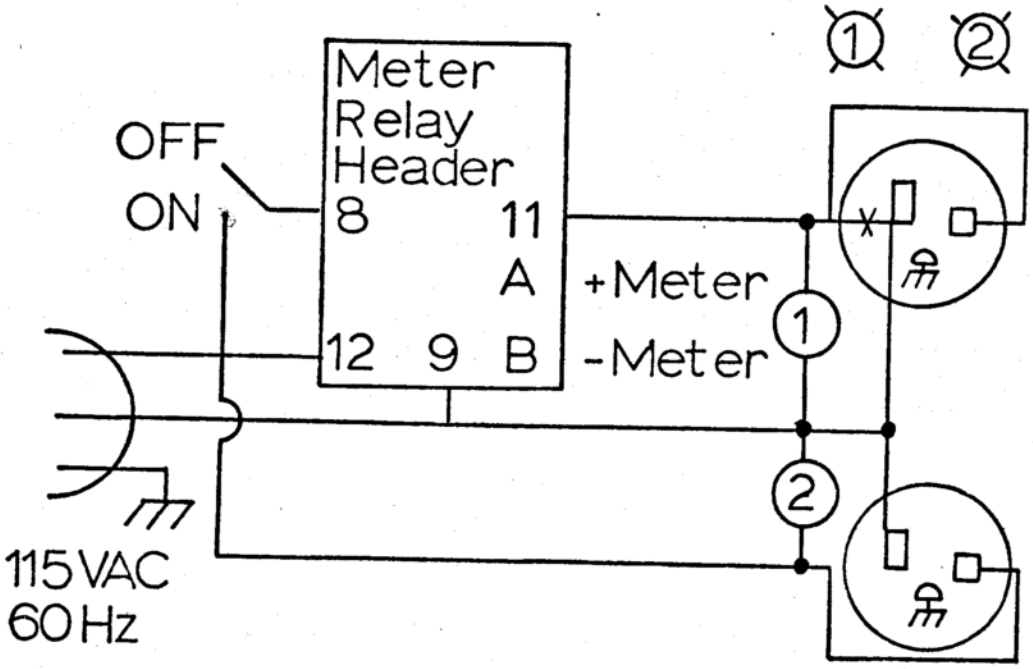

Dissolved Oxygen Tension
(in atmospheres)
0.25
0.20
0.15
0.10
0.05
0.00
TIME
Period of Enrichment
Begin O_2 Enrichment
O_2 Off
Air Sparge Only

FIGURE 5



SIMPSON DUAL LIMIT CONTROLLER
MODEL 3326xA
0-1 MA DC METER RELAY

glycerol (C.P.) 5 gm/l., Bacto agar (Difco) 23 gm/l., and calcium carbonate 5 gm/l. in 1000 ml. of distilled water. The agar was sterilized in 15 ml. aliquots for 20 minutes at 15 pounds pressure, 121°C, in 25 mm by 150 mm culture tubes with plastic closures (Bellco Glass Company). The culture was transferred every two weeks and incubated for 48 hours at 30°C. The culture was also frozen and stored as a suspension in liquid nitrogen.

Streptomyces fradiae 3535 was originally maintained on agar slants of Gould's agar consisting of: Bacto agar (Difco) 20 gm/l., glucose 10 gm/l., yeast extract (Amber 1003) 2.5 gm/l., K_2HPO_4 1 gm/l., in 1000 ml of distilled water. The slants were inoculated with spores of S. fradiae and incubated for seven days at 30°C.

During the course of the study, a problem was observed with the ability of the organism to sporulate on the Gould's agar slants. This inability to sporulate may have caused the lack of reproducibility in the fermentations derived from these slants. Strain 3535 was then transferred and maintained on cobalt-containing Bennett's sporulation agar for streptomyces (40). This agar consisted of: Stadex 60K dextrin (A. E. Staley Company) 10 gm/l., N-Z Amine Type A (Humko-Sheffield Company) 2 gm/l., yeast extract (Amber 1003) 1 gm/l., beef extract (Difco) 1 gm/l., Bacto agar (Difco) 20 gm/l., and $CoCl_2 \cdot 6H_2O$ 20 mg/l. in 1000 ml. of distilled water. Aliquots of 15 ml. of this agar were autoclaved in 25mm by 150mm test tubes with cotton plugs for 30 minutes at 15 pounds of pressure at 121°C. The slants were allowed to cool until hard and were then incubated uninoculated at 37°C for 48 hours in order to dry the surface of the agar. S. fradiae 3535 spores were then inoculated onto the slant which was incubated at 28°C to 30°C. Growth completely covered the agar surface in three days of incubation with 85 to 100 percent of the surface covered

with powdery beige and white spores after an additional 10 to 13 days at 28°C to 30°C. Addition of more than 20 mg/l. $CoCl_2 \cdot 6H_2O$ inhibited growth and sporulation. This culture was transferred every six months followed by incubation for 15 days at 28°C to 30°C.

Bacillus licheniformis 10716 was maintained on agar slants of nutrient agar (Difco) 23 gm/l., 15 ml per 25mm by 150mm tube closed with a plastic closure (Bellco Glass Company). Inoculated slants were incubated at 37°C for 48 hours producing spreading 'lichen-like' colonies. The culture was transferred every month and was also frozen in 12 percent glycerol and stored at -20°C and in liquid nitrogen.

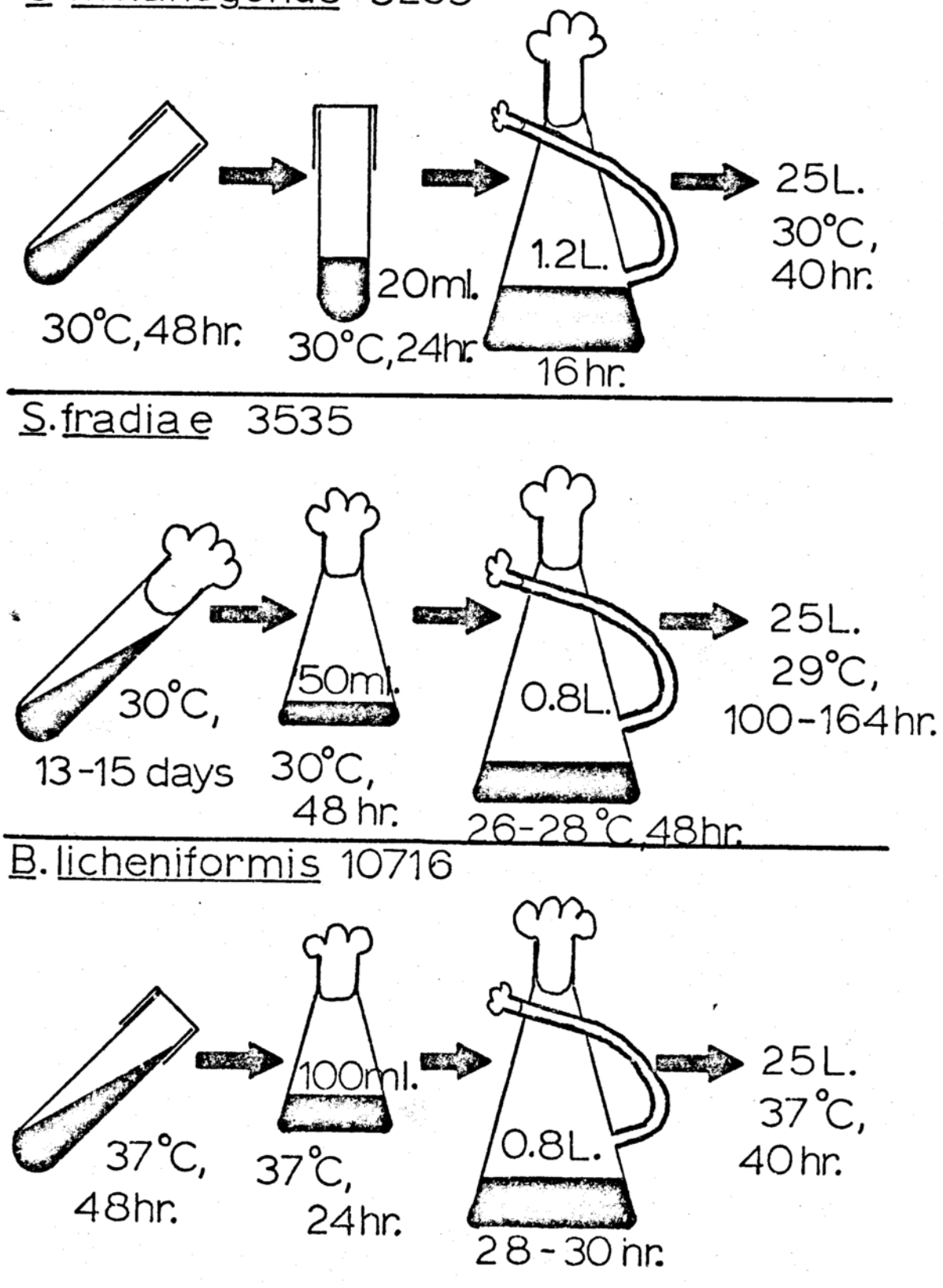
Bacillus subtilis (Marburg), Bacillus subtilis 14593 and Micrococcus flavus 10240-2 were all maintained on Difco nutrient agar slants (23 gm/l.). The slants were autoclaved for 30 minutes at 15 pounds of pressure at 121°C. Inoculated slants were incubated for 48 hours at 30°C for M. flavus, 37°C for B. subtilis strains. The B. subtilis produced cream colored colonies while those of M. flavus were bright yellow. B. subtilis was frozen as a suspension in 12 percent glycerol at -20°C. M. flavus was also frozen at -20°C suspended in nutrient broth (Difco).

E. Inoculum Preparation

The two-stage inoculum preparation procedure for all three fermentations is summarized in figure 6.

The inoculum medium for the growth of G. melanogenus IFO 3293 consisted of: autolyzed yeast extract (Amber 1003 lot number B-4-333) 2 gm/l., glycerol 2 gm/l., and 100 mM pH 7 phosphate buffer. Twenty ml. of this medium in a 25 mm by 150mm tube with a plastic closure (Bellco Glass Co.) was inoculated and incubated at 30°C for 24 hours on a 250 rpm rotary shaker with a one inch displacement (New Brunswick Scientific Company).

FIGURE 6


G. melanogenus 3293
30°C, 48hr.
20ml.
30°C, 24hr.
1.2L.
16 hr.
25L.
30°C,
40hr.
S. fradiae 3535
30°C,
13-15 days
50ml.
30°C,
48 hr.
0.8L.
26-28°C, 48hr.
25L.
29°C,
100-164hr.
B. licheniformis 10716
37°C,
48hr.
100ml.
37°C,
24hr.
0.8L.
28-30 hr.
25L.
37°C,
40hr.

The contents of this tube was then aseptically transferred into 1200 ml. of the same medium in a cotton plugged 4-liter Erlenmeyer flask equipped with a side-arm. A 40 cm length of sterile silicone rubber tube (Silastic^{R} Surgical Grade Tubing) was attached to the side-arm to facilitate aseptic transfer into the fermenter. The 4-liter inoculation flasks were incubated at 30°C in a rotary incubator shaker with a one inch displacement (New Brunswick Scientific Co.) at 250 rpm for 16 hours. The 1200 ml. of inoculum was then aseptically transferred by gravity into the 25 liters of fermentation medium resulting in an inoculum volume of 5 percent of the total volume.

The first stage of inoculum preparation for 25 liter neomycin producing fermentations of S. fradiae 3535 consisted of inoculation of spores into 50 ml. of the following medium: soybean meal (Archer Daniels Midlands Co.) 30 gm/l., glucose 20 gm/l., and calcium carbonate (Fisher C-62) 10 gm/l. The 50 ml. of medium was incubated in a cotton plugged 250 ml. Erlenmeyer flask at 30°C for 48 hours on a 300 rpm rotary incubator shaker. After 48 hours of incubation the culture produced a characteristic blue-green color.

The entire contents of the first stage inoculum flask were transferred into 800 ml. of the same medium contained in a 4-liter side-arm inoculation flask. The inoculation flasks were incubated at 26°C to 28°C for 48 hours on a 300 rpm rotary incubator shaker. The inoculation volume for the S. fradiae fermentations was 3 percent of the total volume.

Inoculum for the bacitracin fermentations was started by inoculation of 100 ml. of nutrient broth (Difco) in a cotton plugged 250 ml. Erlenmeyer flask. This flask was incubated in a 37°C rotary water bath shaker (New Brunswick Scientific Co.) for 24 hours. The entire contents of the first stage inoculum flask was transferred into a 4-liter side-arm

inoculation

flask containing 800 ml. of the following medium: Toasted Nutrisoy Grits 20-40 (63-770 Archer Daniels Midlands Co.) 50 gm/l., sucrose 12 gm/l., $(NH_4)_2SO_4$ 2 gm/l., calcium carbonate (Fisher C-62) 2 gm/l., and 0.05 ml. SAG 5440 silicone antifoam (Union Carbide Company). The inoculum flasks were incubated at 37°C on a rotary incubator shaker at 300 rpm with a one inch displacement for 28 to 30 hours. The inoculation volume for B. licheniformis 10716 fermentations was 3 percent of the total volume.

F. Medium for 25 Liter Fermentations

The medium for all three 25 liter fermentations is summarized in table I. All media were prepared using hot tap water and sterilized in the fermenter for 45 to 60 minutes (depending on the concentration of insoluble material) at 18 pounds, 121°C.

G. melanogenus IFO 3293 fermentations at 30°C were pH controlled at pH 7.0 $\pm$ 0.1 by automatic nonaseptic addition of a 4 N NaoH by a pH controller (New Brunswick Scientific Co.). Foaming was controlled by addition of SAG 4130 silicone antifoam (Union Carbide Co.) prior to sterilization of the fermenter. One hundred twenty ppm of antifoam was required and no antifoam was added during the fermentation.

The pH of the soybean meal - dextrin medium for the growth of S. fradiae after autoclaving was pH 6.5 to pH 6.8 No pH control was used. Foaming was controlled by manual addition of sterile Polypropylene Glycol P-2000 (Dow Chemical) during the 29°C fermentation.

The sucrose of the bacitracin production medium was autoclaved separately dissolved in 2.5 liters of water and aseptically added to the cooled (37°C) sterile fermentation medium. The pH of the complete medium was adjusted to pH 7.0 by aseptic addition of sterile 2 N H_2SO_4.

Table I

Medium for 25 liter fermentations

G. melanogenus IFO 3293	30°C ± 1°C	gm/l
Autolyzed Yeast Extract (Amber 1003 Lot no. B-4-333)		5
$MgSO_4 \cdot 7H_2O$		0.5
KH_2PO_4		8.7
glycerol (C.P.)		15-100
tap water		1000 ml
Antifoam SAG 4130		120 ppm
S. fradiae 3535	29°C ± 1°C	
Soybean meal (Archer Daniels Midlands Co.)		30
Stadex 60K Dextrin (A. E. Staley Company)		30
Antifoam		Polypropylene Glycol P-2000
B. licheniformis 10716	37°C ± 1°C	
Toasted Nitrisoy Grits 20-40 (63-770 Archer Daniels Midland Co.)		50
Sucrose (added separately)		24
$(NH_4)_2SO_4$		2
$CaCo_3$ (Fisher C-62)		2
Antifoam		SAG 5440

B. licheniformis fermentations were not pH controlled. Foaming was controlled by automatic addition of SAG 5440 silicone antifoam (Union Carbide Company) during the fermentation.

G. Microbiological Assays

1. Method for Preparation of Fermentation Samples for Antibiotic Microbiological Assay

Fermentation samples to be bioassayed for neomycin were first adjusted to pH 2 with 6 N H_2SO_4 and placed on a mechanical shaker for 15 minutes (New Brunswick Scientific Co.). The samples were then centrifuged for 30 minutes at 3000 rpm (Sorval Angle Centrifuge) and the pellet of mycelium and soybean meal discarded. The centrifuged samples were adjusted to pH 7 with solid Na_2CO_3 and diluted with 100 mM pH 7.0 phosphate buffer. All samples and neomycin standards were assayed at pH 7.0 diluted in phosphate buffer.

Bacitracin containing fermentation samples were first centrifuged for 30 minutes at 3000 rpm (Sorval Angle Centrifuge) and then diluted 5 or 10 fold with 50 mM pH 6.5 phosphate buffer. All samples and standards were assayed diluted in this pH 6.5 buffer. In some cases, inhibition zones became overgrown with B. licheniformis causing difficulty in reading of the zone diameter. These samples could be 'sterilized' by filtration through 0.45 micron type HA membrane filters (Millipore Filter Corporation) without detectable antibiotic (less than 0.1 unit/ml.) adhering to the filter.

2. Agar Diffusion Microbiological Assay for Neomycin and Bacitracin

The bioassays for neomycin and bacitracin were in 21 cm by

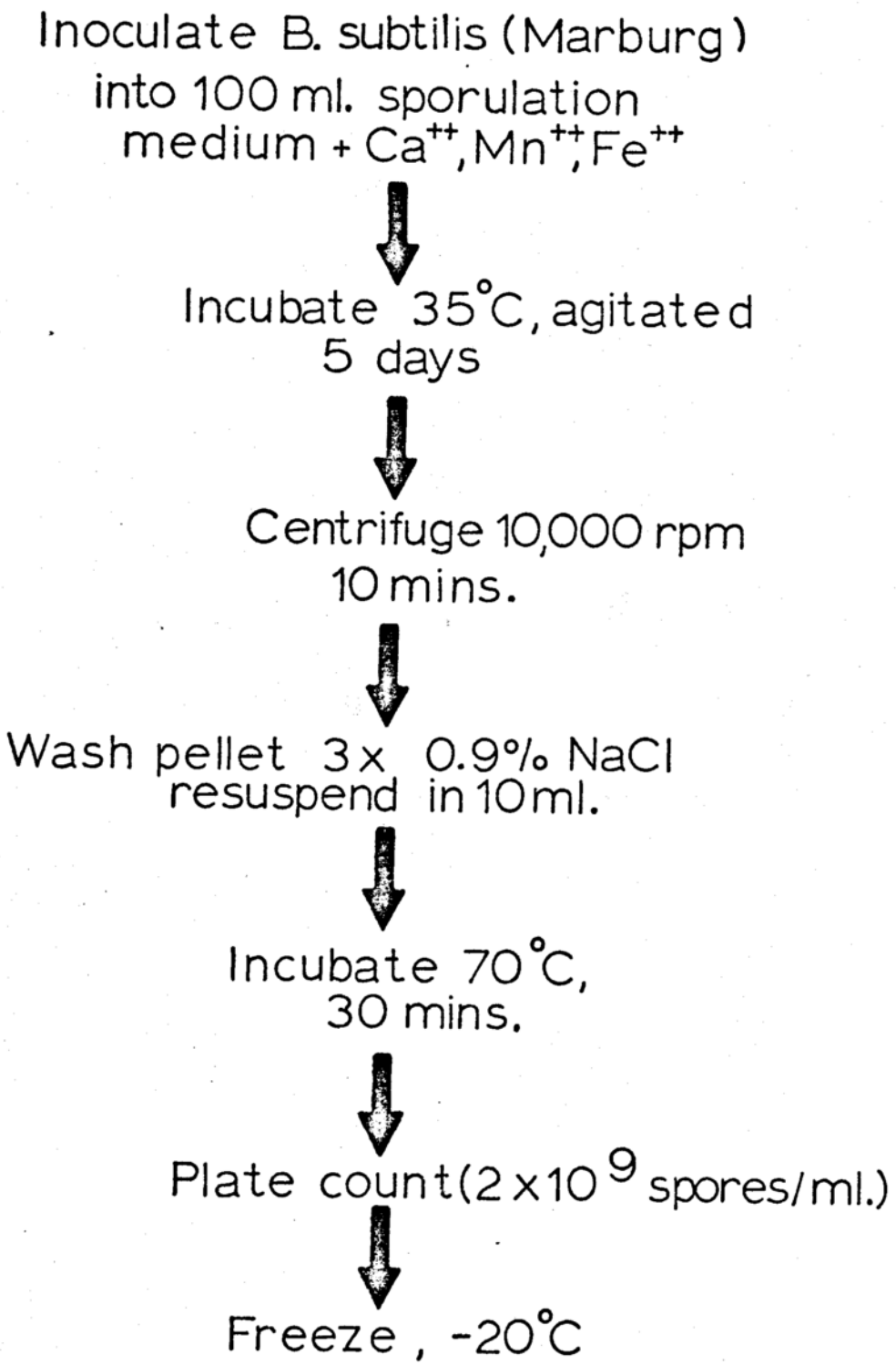
35 cm Pyrex baking dishes with removable stainless steel covers.

Neomycin was assayed by a paper disc plate method (41,42) using a spore suspension of B. subtilis (Marburg) frozen in 0.9 percent NaCl. The method used for preparation of frozen (-20°C) spore suspensions is summarized in figure 7.

A two layer agar system was employed to enhance the sensitivity of the assay. Two hundred ml. of Difco Antibiotic Assay Medium number 11 (pH 7.9) was used for the base layer. The top agar layer consisted of 70 ml. of the same agar inoculated with 2 ml. of a 10^7 spores/ml. suspension of B. subtilis thawed at 37°C. Agar for the top layer was maintained at 50°C until inoculation.

Neomycin antibiotic test solutions (pH 7.0) were drawn up into 6.35 mm paper discs (Schleicher and Schuell) by capillary action and placed on the surface of the inoculated solidified agar. Each Pyrex dish could hold up to 50 discs without overlapping inhibition zones. Of these 50 discs on each plate, at least 13 were of known concentrations of neomycin sulfate standard (S. B. Penick lot no. 53-NHF-1, 675 micrograms free base per milligram). Plates of B. subtilis were incubated at 37°C for 20 hours (National Incubators). The antibiotic inhibition zones were read manually using a vernier caliphers graduated in 0.1 mm divisions. The average value of three discs was used for each determination. Data were plotted as the logarithm of the antibiotic concentration in micrograms per ml. against the square of the observed zone diameter in millimeters. A separate curve was run for each plate and the slope of the line determined by the response of the standards on that plate. The accuracy of this

FIGURE 7


Inoculate B. subtilis (Marburg) into 100 ml. sporulation medium + Ca^{++}, Mn^{++}, Fe^{++}
Incubate 35°C, agitated 5 days
Centrifuge 10,000 rpm 10 mins.
Wash pellet 3x 0.9% NaCl resuspend in 10 ml.
Incubate 70°C, 30 mins.
Plate count (2×10^9 spores/ml.)
Freeze, -20°C

assay was ± 15 percent which is within expectations of a bioassay of this type.

Bacitracin was assayed using a suspension of Micrococcus flavus 10240-2 stored frozen in nutrient broth (Difco). A similar two layer agar system was employed as for the neomycin bioassays except that Difco Antibiotic Assay Medium number 1 (pH 6.5) was employed. The top agar layer was inoculated with 2 ml. of a frozen suspension of M. flavus thawed at 37°C. Antibiotic test solutions containing bacitracin were assayed using a cylinder-plate method (43). Low concentrations of bacitracin adhere to paper reducing the sensitivity of the assay if paper discs are used as reservoirs. Ten mm by 6 mm sterile stainless steel cylinders (Fisher Pencylinders) were placed on the agar as soon as it solidified.

The cylinders were filled with 100 microliters of bacitracin test solutions diluted in pH 6.5 phosphate buffer. One hundred microliter disposable pipets were used to deliver the solutions into the cylinders (Microcaps). The plates were incubated at 30°C for 24 hours and the inhibition zones read using a vernier caliphers. Three cylinders were used for each determination. Each Pyrex plate contained up to 30 cylinders, 12 of which contained known concentrations of bacitracin standard (S. B. Penick lot no. 414-NEF-1, 50.2 units per milligram). The results of each bioassay plate were plotted as the square of the inhibition zone diameter against the logarithm of the concentration of bacitracin in international units/ml. (44) The same ± 15 percent accuracy was observed for this assay as for the neomycin bioassay.

Standard response curves for the neomycin disc-plate bioassay

and the bacitracin cylinder-plate bioassay are shown in figures 8 and 9.

3. Bioautography of Bacitracins

Compounds extracted by a neutral n-butanol extraction of bacitracin fermentation beer were separated by thin layer chromatography on 5 cm by 20 cm precoated plastic silica gel plates (Eastman 6061) and assayed for antibiotic activity by bioautography.

Forty microliters of n-butanol extract was spotted on each of two spots on the 5 cm by 20 cm plate. The chromatogram was developed in a n-butanol-acetic acid-water (4:1:2) solvent system for four hours. The developed chromatogram was dried at room temperature for two days and then placed in a jar containing 6.5 N ammonia vapor for ten minutes in order to neutralize any remaining acetic acid. The thin layer plate was cut in half lengthwise and one half sprayed with ninhydrin spray to detect the position of the separated compounds. The areas of the chromatogram corresponding to the ninhydrin positive spots were cut from the unsprayed half and placed silica gel side down on a bioassay plate inoculated with M. flavus. The pieces of the chromatogram remained in contact with the agar surface for 90 seconds. After removing the chromatogram pieces, the bioassay plate was incubated for 24 hours at 30°C. Pieces of the chromatogram containing compounds having antibiotic activity against M. flavus produced zones of growth inhibition. Chromatograms of standard mixtures of bacitracin A and B were run along with beer samples in order to determine the mobility of bioactive bacitracins in this system.

FIGURE 8

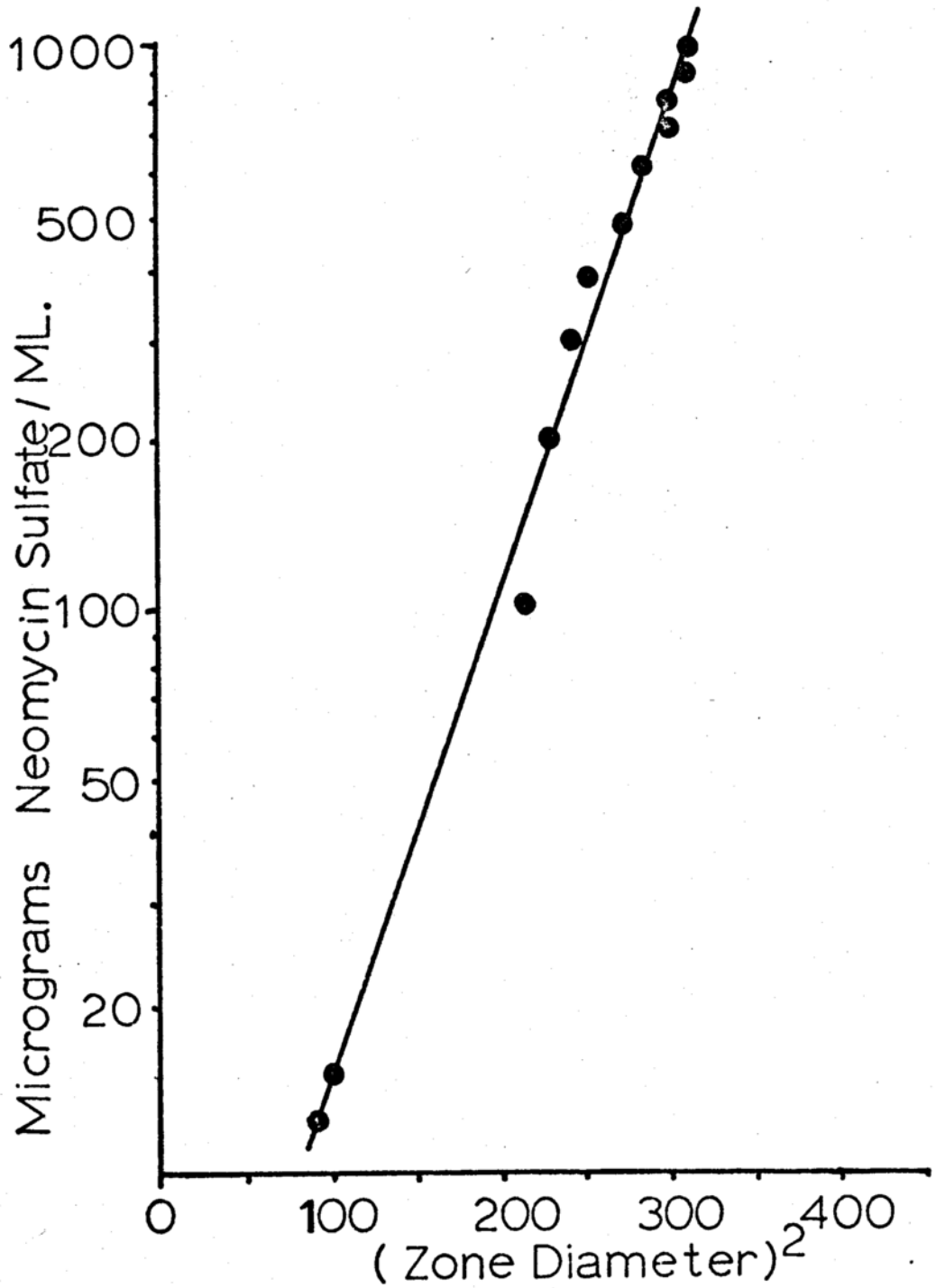
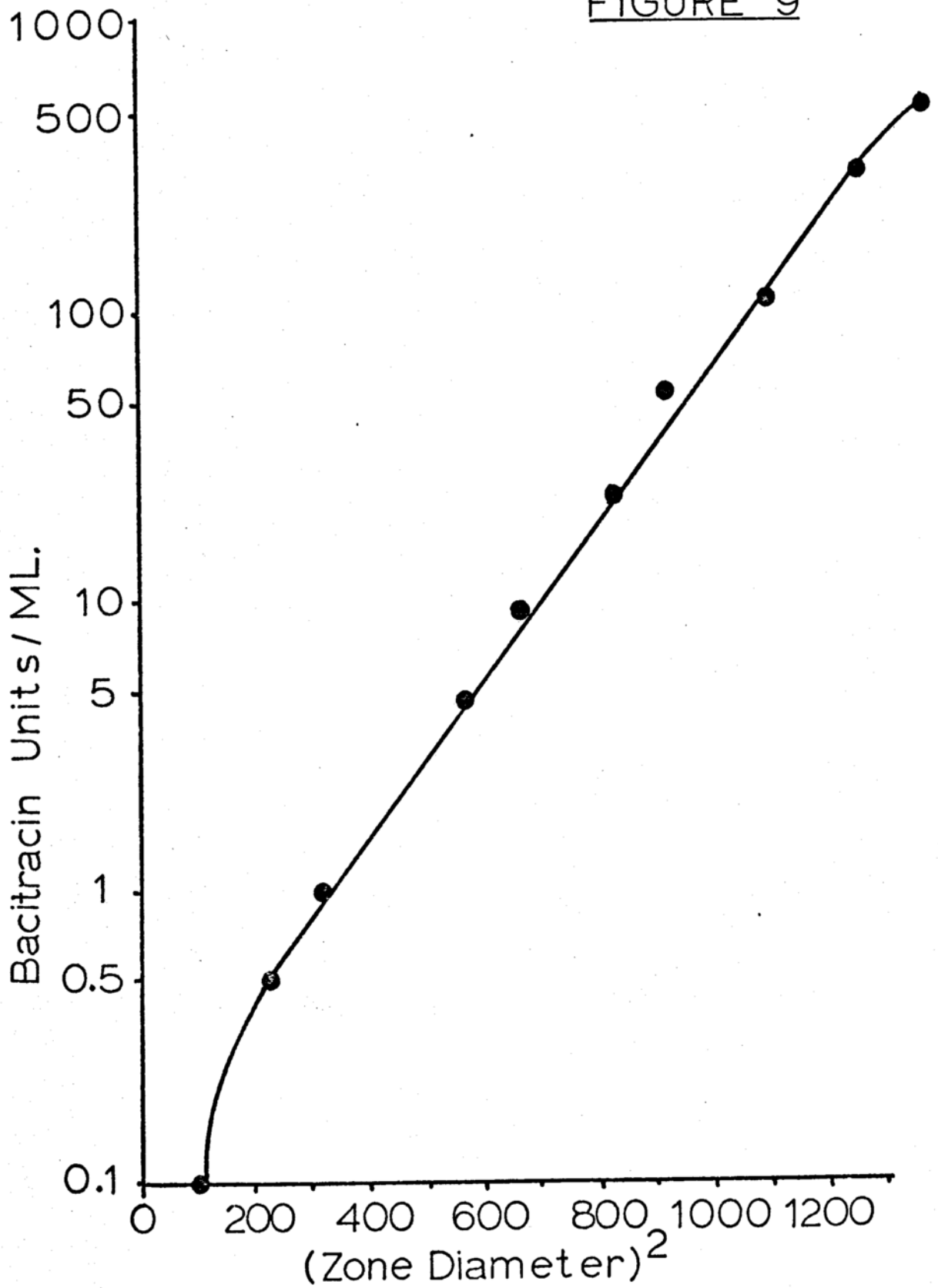

1000
500
200
100
50
20
Micrograms Neomycin Sulfate / ML.
0
100
200
300
400
(Zone Diameter)^2

FIGURE 9


1000
500
100
50
10
5
1
0.5
0.1
Bacitracin Units / ML.
0
200
400
600
800
1000
1200
(Zone Diameter)^2

H. Chromatographic Methods

1. Paper Chromatography

a. Identification of Dihydroxyacetone

The identity of dihydroxyacetone (DHA) found in the fermentation of G. melanogenus was confirmed by ascending chromatography on the following solvent systems: 1) n-butanol-pyridine-water (10:3:3) DHA R_f=0.72, 2) ethyl acetate-pyridine-water-acetic acid (glacial), (5:5:3:1) DHA R_f=0.81, 3) n-butanol-ethanol (95 percent)-water (40:11:19) DHA R_f=0.56, and 4) water-saturated phenol DHA R_f=0.87. In all four systems the relative mobility of DHA extracted from the fermentation medium with ethylacetate was identical to that of standard compound (Aldrich Chemical Co.). DHA was located on the paper chromatograph with either a two percent methanolic solution of triphenyltetrazolium chloride or a 0.2 percent solution of o-phenylenediamine in one percent ethanoloic nitric acid followed by heating for 45 minutes at 65°C. DHA produced a red spot on a colorless background when visualized with the tetrazolium chloride spray and a violet-gray spot when employing the o-phenylenediamine reagent.

b. Detection of Neomycin C

Paper chromatography was used as a preliminary screening technique in order to detect neomycin C in fermentation samples. The method of separation of N-acetylated neomycins was used (45).

To an aqueous sample containing 1-2 mg of neomycin mixture 0.1 ml of 0.3 M K_2HPO_4 plus 0.1 ml acetic anhydride was added followed by thorough mixing. Forty microliters of this sample was spotted on a 38 cm by 24 cm sheet of Whatman number 1 chromatography paper for descending development in a n-butanol-pyridine-

water (60:40:30) solvent system. The chromatogram was developed for 24 hours at room temperature. Neomycin B and C were detected using the following method. The chromatogram was dried at 50°C and sprayed with a hypochlorite reagent (1:20 v/v Chlorox in water). The chromatogram was again dried at room temperature until no hypochlorite odor could be detected. The paper was then sprayed with 95 percent ethanol and allowed to dry at room temperature. Following a starch-iodine spray (1:1 v/v 1 percent KI and 1 percent soluble starch) N-acetylated neomycins appeared as dark blue spots on a white background. The R_f of neomycin B and C after 24 hours of development was 0.47 and 0.32 respectively.

c. Detection of Neamine

Neamine (Neomycin A) was also detected in fermentation samples by using paper chromatography on Whatman number 1 (46). In this system the neomycins are chromatographed without derivitization. The descending chromatograph was developed in a solvent system consisting of: methyl ethyl ketone-isopropanol-6.5 N ammonium hydroxide (80:20:30) for 22 hours. The compounds were detected by spraying with ninhydrin reagent (0.25 percent ninhydrin in 95 percent ethanol) followed by heating at 110°C for 10-15 minutes. The neomycins appeared as light blue spots on a white background. The relative mobility of these compounds (R_f) after 22 hours of development was: neamine, 0.40-0.45; neomycin B, 0.26; and neomycin C, 0.17.

d. Identification of Sucrose, Glucose, and Fructose

Sucrose, glucose, and fructose present in fermentation medium were detected by paper chromatography on Watman number 1 paper using the following solvent system: (47) n-butanol-pyridine-water (3:1.3:1.5). The chromatogram was spotted with 40 microliters of

centrifuged fermentation beer and developed for 24 hours in a descending method. The chromatograms were dried at room temperature. Sugars were detected with aniline phthalate spray (48) (930 mg aniline + 1.6 gm phthalic acid in 100 ml. of water saturated n-butanol) producing brown spots on a light background. They could also be detected by an anthrone reagent spray (49) (300 mg. anthrone (Baker Chemical Co.) dissolved in 10 ml. glacial acetic acid, followed by addition of 20 ml. of ethanol, 3 ml. H_3PO_4, and 1 ml. water). When the chromatograms sprayed with this reagent are heated for three to five minutes at 110°C, the sugars produce yellow to brown spots on a white background. Longer heating (10-15 minutes) is required to detect fructose using the anthrone spray. The sugars produced the following relative mobility with respect to sucrose ($R_{sucrose}$): sucrose 1.0, glucose 1.6, fructose 1.4, olligo-saccharides remained at the origin.

2. Thin-Layer Chromatographic Methods

a. Separation of Neamine, Neomycin B and C

The neomycins could also be separated without derivitization using 15 cm by 20cm silica gel GF coated glass plates without heat activiation. (Analtech, 250 micron) The plates were developed in methanol-concentrated ammonium-hydroxide(4:1:5) for 3.25 hours and the compounds detected by ninhydrin spray followed by heating at 90°C for 10 minutes. The following R_f values were observed: neamine 0.35, neomycin C 0.16, and neomycin B 0.14.

b. Separation of Bacitracins

The bacitracin antibiotics and the bacitracin-like compounds

produced in the fermentation of B. licheniformis could be separated by thin-layer chromatography on 15 cm by 20 cm silica gel GF plates (Analtech, 250 micron) and a n-butanol-acetic acid-water (4:1:2) solvent system (50). Forty micro-liters of neutral n-butanol fermentation extracts containing 20 mg per ml. of bacitracins were spotted and the thin-layer chromatography plates developed for four hours. The plates were then dried and sprayed with ninhydrin spray followed by heating at 110°C to detect the compounds. Bacitracin A and B produced a yellow-orange spot with R_f=0.41, Bacitracin X produced a dark purple spot of R_f=0.64 and Bacitracin F showed a pink color of R_f=0.47. The presence of bacitracin A and B on the chromatogram could also be detected by ultra violet fluorescence. (Chromato-Vue, Ultra-Violet Products Inc.)

3. Column Chromatographic Separation of Neomycin B and Neomycin C

Column chromatographic separation of neomycin B and C was used in order to obtain sufficient pure B and C for use as standards for gas-liquid chromatography.

A 1 cm by 75 cm Dowex 1 (OH^- form) column (200-400 mesh) separated neomycin B from C contained in a commercial mixture of neomycins (S. B. Penick lot No. B-V-697997-6). The column was charged with 300 mg. of neomycin sulfate mixture in 1 ml. of distilled water. Elution of the column was with deionized water degassed by autoclaving. Detection of elution of neomycins was by paper chromatographic methods already described for separation of B and C. One hundred and twenty 3-4 ml. fractions were collected.

Neomycin C eluted in fractions number 30 to 55, and neomycin B eluted in fractions number 90 to 120. Fractions 55 to 90 contained both neomycins. Forty-three mg of pure lyophylized neomycin B and 26 mg of pure neomycin C were recovered. No neamine was present in this commercial mixture.

4. Determination of the Ratio of Neomycin B to C by Gas-Liquid Chromatography

a. Sample Preparation

Fermentation samples were prepared for gas-liquid chromatography analysis of neomycins by adding 20 ml. of centrifuged beer (pH 7) to the top of a 1.2 cm by 2.5 cm column of wet Amberlite CG-50 resin (100-200 mesh) in the NH_4^+ cycle. After the 20 ml. sample had passed through the resin, the column was washed three times with 10 ml. of distilled water. Neomycins were eluted from the column with 10 ml. of 1 N NH_4^+ and lyophylized overnight. (New Brunswick Scientific Company, Cryolyzer Freeze-Dryer). The CG-50 columns were regenerated for re-use by addition of 5 ml. of concentrated NH_4OH followed by 20 ml. of 1 N NH_4OH

b. Derivativization of Neomycins

Preparation of volatile neomycin derivatives and gas-liquid chromatography followed the methods of Tsuji (51) and Omoto (52):

The lyophylized samples were derivatized in 1.5 ml. serum bottles with red rubber closures. The entire lyophylized sample from the 10 ml. of CG-50 column eluate was added to the serum bottle. One half ml. of Tri-Sil Z (Pierce Chemical Company) and 80 microliters of N-Trimenthylsilyl-diethylamine (TMSDEA) (Pierce

Chemical Company) were added and the sealed vial heated at 75°C for 40 minutes. Samples were derivatized and analyzed on the same day for best results. Derivatized day-old samples stored -20°C could be used by addition of an additional 80 microliters of TMSDEA followed by heating at 75°C for 20 to 30 minutes.

c. Gas-Liquid Chromatography Conditions

Gas-liquid chromatography of the derivatized samples was done on a Barber Colman Series 5000 Gas Chromatograph. A six foot U-shaped glass column equipped with red rubber septa was packed with 0.75% OV-1 (on Gas Chrome Q, 100-200 mesh) column packing (Applied Scientific Labs Inc.). The column was non-flow conditioned for 12 hours at 310°C then pretreated by injection with 50 microliters of Silyl-8 (Pierce Chemical Company) in 5,10 microliter injections.

The separation of neomycin B and C could by obtained under isothermal conditions at 300°C with carrier gas flow (N_2) at 66 psig, injector temperature 300°C and detector temperature at 360°C. Under these conditions the retention times of neomycin B and C were 3.0 and 3.6 minutes respectively. Neamine if present came off of the column in the solvent peak under these conditions.

Neomycin B and C could also be separated by temperature programming the column oven from 250°C to 310°C at approximately 2.5°C per minute. Under these conditions the retention times of Neomycin B and C were 11.2 minutes and 12.6 minutes.

Neamine could be separated from neomycin B and C if the temperature program started from 190°C and ran to 310°C. Under these conditions, the retention times were: neamine 5-6 minutes, neomycin B, 13 minutes, and neomycin C, 14 minutes.

d. Quantitation of the Ratio of Neomycin B to Neomycin C

Quantitation of the ratio of neomycin B to C was performed by two methods: peak height, or photo-copying the GLC trace and cutting and weighing the neomycin B and C peaks.

For the peak height method, the GLC response of pure neomycin B and pure neomycin C was determined by repeated 2 microliter injections of a 1.83 mg/ml. derivatized solution of neomycin B and a 1.50 mg/ml. derivatized solution of neomycin C. Seven injections were made of each standard solution using the 250°C to 310°C temperature program conditions (2.5°C/minute, attenuation setting 2×10^{-8}) followed by measuring the peak height. Discarding the highest and the lowest values, the average peak height per microgram of antibiotic was determined. The GLC response of neomycin C was determined to 0.81 that of an equal weight of neomycin B using the above method.

5. Determination of the Ratio of Bacitracin A to B by High Pressure Liquid Chromatography

a. Sample Preparation

Samples for high pressure liquid chromatographic analysis of bacitracins were prepared from 1 liter of culture sampled at the end of each B. licheniformis fermentation. Approximately 300 gms. of filter aid (Johns-Manville, Celite 503) was added to the 1 liter sample with stirring. The slurry was filtered and washed with water under vacuum. The cake was discarded. The filtrate (approximately 1500 ml) was adjusted to pH 7 with 6 N H_2SO_4 followed by two extractions each of 250 ml. of n-butanol. Centrifugation was required to

break the emulsion formed during extraction. The aqueous phase was discarded and the solvent layer concentrated to approximately 100 ml. under vacuum at 40°C. The antibiotic precipitated as the n-butanol extract was concentrated. This precipitate was collected on a 0.45 micron membrane filter (Millipore), dried at room temperature for 24 to 48 hours, and dissolved in 5 ml. distilled water. Twenty to forty units (40 to 100 microliters) were injected for analysis of bacitracin A, B, and F.

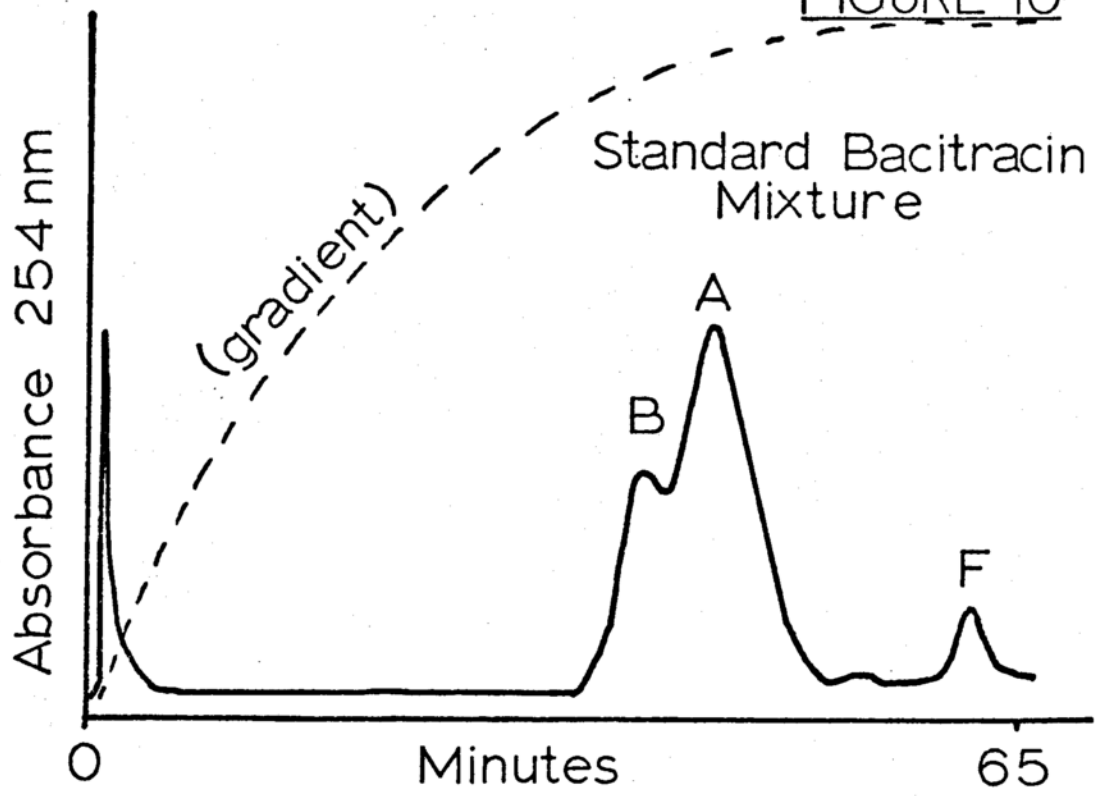
b. Column condition

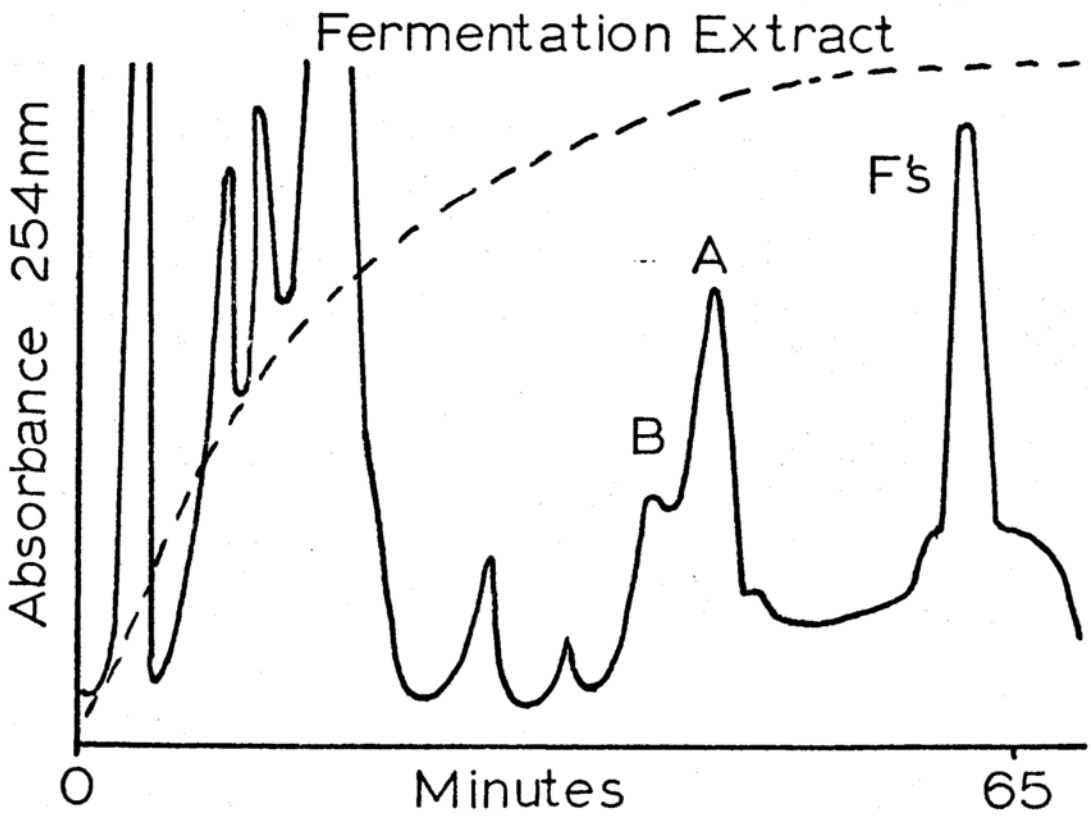
High pressure liquid chromatographic analysis of bacitracin fermentation samples for the ratio of A to B produced were analyzed by a modification of the method of Tsuji (53,54). A four foot Bondapak C_{18}/Corasil column was used with a programmed convex gradient elution of decreasing polarity, from five percent methanol in pH 4.5 buffer to 40 percent methanol and 20 percent acetonitrile in pH 4.5 buffer. The flow rate of the column was 1.5 ml./minute with an elution time of 65 minutes at 1000 psig pressure. Detection of the eluting compounds was made using an ultra violet detector at 254 nm.

c. Separation of bacitracins

Bacitracin A, B, and F could be separated and quantitated, by the method of peak height, using IMC lot. no. 80171-2 bacitracin A, B, and F mixture as standard. (figure 10) Also shown in figure 10 is the HPLC trace from a fermentation sample demonstrating the separation of the bacitracins from other medium components extracted at neutral pH with n-butanol.

FIGURE 10


Standard Bacitracin Mixture
(gradient)
Absorbance 254 nm
A
B
F
0
Minutes
65


Fermentation Extract
Absorbance 254nm
F's
A
B
0
Minutes
65

I. Determination of Cell Dry Weight

1. Method for Soluble Media

The growth of G. melanogenus in soluble media was followed by correlating the absorbance of washed resuspended cell dry weight.

A 200 ml 20 hour culture was grown on the inoculum medium (page 27) in one 2000 ml. Erlenmeyer shaken flask at 30°C. The cells were harvested by centrifugation, washed twice in pH 7 100 mM phosphate buffer and resuspended in the same. Aliquots of the cell suspension were diluted with buffer and the absorbance at 650 nm recorded on a Bausch and Lomb Spectronic 20 spectrophotometer. The aliquots were then placed in pre-weighed and dried aluminum weighing pans and dried at 100°C for 15 hours. The dried cells were allowed to cool in a dessicator and weighed on an analytical balance. Aliquots of the cell suspension were adjusted so that at least 30 mg. dry weight of cells were weighed in each pan. Triplicate determinations were made at each dilution. The cell dry weight was corrected for the weight of the buffer salts.

Cells from fermentation samples were washed and resuspended in pH 7 100 mM phosphate buffer. The absorbance of these samples at 650 nm was then compared with the previously determined linear correlation between cell dry weight and absorbance. An absorbance of 1.00 at 650 nm represented 670 mg per liter of G. melanogenus cell dry weight.

2. Method for Media Containing Insolubles

A direct dry weight method was devised in order to determine the approximate cell mass produced during fermentations of S. fradiae and B. licheniformis growing on media containing insoluble protein sources

(soybean meal, soy grits) and calcium carbonate.

Ten ml. of culture was filtered through a glass fiber filter under vacuum and the filter washed with 20 ml. of water. The filtrate was adjusted to pH 2 to pH 3 to solubilize calcium carbonate if present. The cell-laden liquid was then centrifuged for 30 minutes at 3000 rpm (Sorval Centrifuges) and the supernatant discarded. The cell pellet was washed with 10 ml. of 50 mM pH 7 phosphate buffer and resuspended in the same. The washed cells were quantitatively transferred to a pre-weighed and dried aluminum weighing pan and dried at 100°C for 15 hours. The dried cells were allowed to cool and weighed on an analytical balance. The weight of the buffer salts was subtracted from the dry weight. Each direct dry weight determination was done in duplicate.

3. Comparison of Dry Weight Methods

Comparison dry weight determinationswere performed on 1800 ml. of a 17 hour culture of B. licheniformis (200 ml. per 2 liter Erlenmeyer shaken flasks) grown at 37°C on nutrient broth (Difco). The cell dry weight of 900 ml. was determined by each method in four determinations weighing between 35 and 45 mg. of cell dry weight in each. The method for determination of cell dry weight for insoluble media appeared to give cell mass values 30 percent higher than the method of simply washing the cells with buffer. This difference could be due to precipitation of soluble protein from the medium during the pH drop used to solubilize calcium carbonate. This 30 percent error was considered acceptable for estimation of cell growth on culture media containing insolubles.

J. Determination of Sporulation of Bacilli

The percentage of cells sporulating during fermentations of B. licheniformis 10716 and B. subtilis 14593 was followed by a spore stain according to Bartholomew and Mitwer (55).

Two microscope slides were prepared for each fermentation sample and stained. The percentage of cells sporulating was determined by counting two microscopic fields of at least 50 cells twice on each slide using an oil emersion (970X) light microscope (American Optical Company, Model 50). The average of these four determinations was taken as the approximate percentage of cells sporulating.

K. Chemical Assays

1. Determination of Dihydroxyacetone

Dihydroxyacetone was determined colorimetrically by a modification of the method of Campbell (56). One tenth ml. of centrifuged fermentation medium containing less than 150 micrograms of dihydroxyacetone was combined in a 12 mm by 150 mm test tube with 0.5 ml. of a phosphate molybdate reagent (56).

The contents of the tube were mixed on a Vortex mixer (Scientific Industries Inc.) and placed in a boiling water bath for 15 minutes. The tubes were then removed from the bath and allowed to cool to room temperature. The samples were diluted with 10 ml. of distilled water and the blue color recorded at 660 nm on a Bausch and Lomb Spectronic 20 spectrophotometer.

This assay produced a linear response between 0 and 800 micrograms of anhydrous dihydroxyacetone dimer (Aldrich Chemical Co.) The precision of this method was $\pm$ 2 percent.

2. Identification of Dihydroxyacetone

The infrared spectrum of isolated DHA, extracted from the fermentation medium by ethyl acetate, was compared with standard DHA dimer (Aldrich Chemical Co.) on a Perkin Elmer 324 Infrared Spectrometer. The absorption frequencies of the isolated and standard DHA were found to be identical.

3. Anthrone Assay for Carbohydrates

The anthrone assay for carbohydrates (57) was used for determination of residual dextrin, $(C_6H_{10}O_5)_n \cdot xH_2O$, in S. fradiae fermentations and for residual sucrose in B. licheniformis fermentations.

Fermentation samples containing 30 gms/l. of Stadex 60 K dextrin (A. E. Staley) were centrifuged for 30 minutes at 3000 rpm and 0.1 ml. of the clear supernatant diluted in 10 ml. of distilled water. One tenth ml. of this dilution was assayed in duplicate for residual dextrin.

One ml. of centrifuged broth containing 24 gms/l. sucrose was diluted with 100 ml. of distilled water. One tenth ml. of this dilution was assayed in duplicate for residual sucrose.

In the anthrone assay, 0.1 ml. of sample is pipetted into a 12 mm by 150 mm test tube cooled in ice water. Five ml. of freshly prepared anthrone reagent (5 ml. ethanol, 200 mg anthrone (Baker Chemical) made up to 100 ml. in 75% concentrated H_2SO_4) is added with mixing on a Vortex mixer (Scientific Industries Inc.). The tubes are then incubated for 10 minutes in a vigorously boiling water bath and returned to the ice bath. When cool, the absorbance of each tube was recorded at 625 nm on a Bausch and Lomb Spectronic 20 spectrophotometer.

The anthrone assay produced a linear response between 0 and 500 micrograms per ml. with Stadex 60 K dextrin with an absorbance at 625 nm of 0.51 equal to 400 micrograms per ml.

The same assay produced a linear response between 0 and 30 micrograms per ml. of sucrose (reagent grade) with an absorbance of 0.49 equal to 30 micrograms per ml. sucrose assayed. The precision of this assay was between ± 2 and ± 5 percent.

4. Determination of Lactic Acid

The method used for determination of lactic acid was that of Barker and Summerson (58). Centrifuged fermentation samples were diluted 10 fold with water and duplicate 0.1 ml. samples containing 0-10 microgram per ml. lactic acid assayed. Six ml. of cold concentrated H_2SO_4 (reagent grade) was added to the 0.1 ml. sample in 12 mm by 150 mm test tubes in an ice bath. The tubes were swirled to mix, incubated for 5 minutes in a vigorously boiling water bath, and returned to the ice bath. One drop of four percent $CuSO_4 \cdot 5H_2O$ solution was added with 0.02 ml. of p-hydroxydiphenyl reagent (1.5 gm p-hydroxy-diphenyl dissolved in 100 ml. of 0.5 percent NaOH). After mixing, the tubes were incubated at room temperature for 10 minutes in order to develop the purple color. The tubes were placed into the boiling water bath for 90 seconds, removed, and allowed to cool at room temperature. The absorbance of each tube at 565 nm was recorded on a Bausch and Lomb Spectronic 20 spectrophotometer.

This assay produced a linear response between 0 and 80 micrograms anhydrous L(+) lactic acid (Sigma Chemical Company), however the region of greatest precision (± 4 percent) was between 0 and 10 micrograms assayed. An absorbance of 1.00 at 565 nm equals 83 micrograms of anhydrous L(+) lactic acid assayed.

L. Method for Study of the Nutritional Requirements of G. melanogenus 3293

The study of the nutritional requirements of G. melanogenus was undertaken using 50 ml. of medium in 250 ml. cotton plugged erlenmeyer flasks on a 30°C rotary shaker at 300 rpm (New Brunswick Scientific Company). Incubation of the inoculum for these studies was for 48 hours in the inoculum medium previously described. 0.1 ml. of the inoculum culture was used to inoculate each 50 ml. of test medium. Growth of the cultures was recorded as the change in the absorbance of the washed cells of the culture in 48 hours of incubation at 30°C. Each test was run in triplicate, and the average of the three values reported.

M. Methods for Determination of the Oxygen Transfer Rate of the Fermenter

1. Sulfite Oxidation

The ability of the 50 liter fermenter to transfer oxygen into distilled water at 30°C was measured by the method of sulfite oxidation (59). This method is based on the following reaction in aqueous solution:

$$Na_2SO_3 + \tfrac{1}{2}O_2 \rightarrow Na_2SO_4 \qquad \text{(viii)}$$

The fermenter was filled with 25 liters of distilled water, the temperature allowed to equilibrate to 30°C, and three-tenths normal Na_2SO_3 added. The impeller speed was adjusted to the desired rpm and the Na_2SO_3 mixed until completely dissolved (2-5 minutes). Sufficient $CuSO_4$ was then added to make the final solution at least 10^{-3} molar and stirred for 30 seconds before aeration was begun. Samples were taken directly from the fermenter (through drain valve) every five minutes for 25 to 35 minutes. Five milliliters of the sampled solution was added to a 250 ml. erlenmeyer flask containing 25 ml. of 0.1 N

plus 5 ml. of 18 percent HCl (v:v). The excess iodine was titrated with standard 0.1 N $Na_2S_2O_3$ using a starch endpoint.

The milliliters of standard thiosulfate required to titrate the excess iodine plotted against the time produced a straight line with a positive slope. The oxygen transfer rate (K_d), and the volumetric oxygen transfer coefficient determined by sulfite oxidation (K_La_s), were calculated from the slope of these determinations using the following formula:

$$K_d = \frac{1}{P_G}\frac{dc}{dt} = \frac{-\frac{1}{4}\left(\frac{0.1}{d}\right)\left(\frac{da}{dt}\right) f_{S_2O_3} \times 10^{-3}}{P_G(0.21)} \quad \frac{\text{gm. mole } O_2}{\text{ml. min. atm.}} \qquad \text{(ix)}$$

$$K_La_s = \frac{K_d}{H} = \frac{10^{-3} \times 60 \times K_d}{H} \quad (hr^{-1}) \qquad \text{(x)}$$

where K_d = oxygen transfer rate in gm. moles O_2/ml. min. atm.

K_La_s = maximum volumetric oxygen transfer rate determined by sulfite oxidation in hr^{-1}

d = sample volume (ml.)

P_G = partial pressure of oxygen in air at 30°C (atm.)

$f_{S_2O_3}$ = standardized concentration of thiosulfite solution

$\frac{da}{dt}$ = slope of the determination of ml. of standard S_2O_3 required to titrate excess iodine per minute of determination

H = Henry's constant for oxygen dissolved in distilled water at 30°C with 1 atmosphere pressure

The value of K_La_s determined represents the maximum value of the volumetric oxygen transfer coefficient (for distilled water, 30°C, atmospheric pressure)

attainable under the specified conditions of aeration and agitation. They do not represent actual K_La values in the fermentation medium during the fermentation. This determination was used only to compare the effect of varying of the physical factors of impeller speed and aeration rate on the oxygen transfer ability of a particular piece of fermentation equipment.

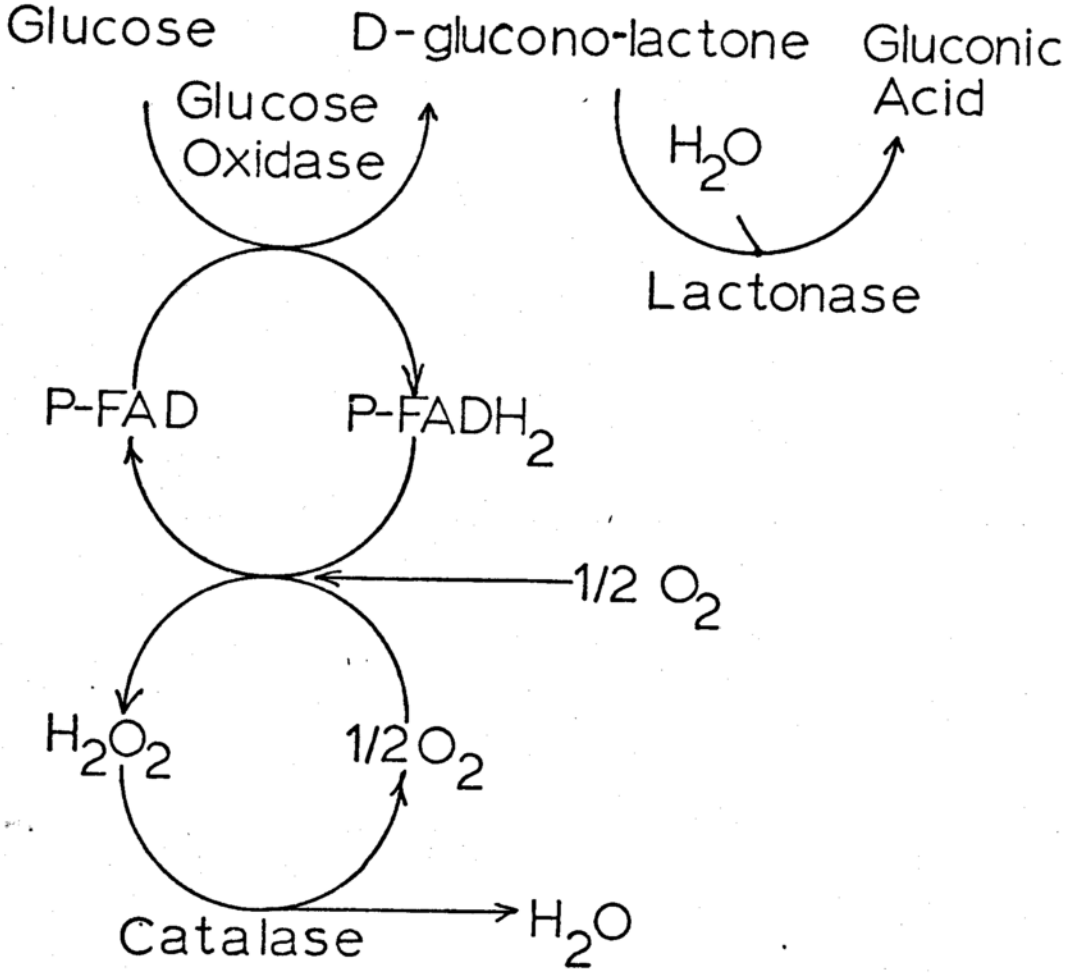
2. Glucose Oxidase

The effectiveness of aeration of a fermentation vessel (oxygen transfer rate) can also be determined enzymatically using a glucose oxidase system to simulate the oxygen transfer in fermentation broth (60). The mechanism of enzymic reaction of the glucose oxidase system in shown in figure 11.

The 50 liter fermenter equipped with a dissolved oxygen probe is charged with 25 liters of 0.1 molar sodium phosphate buffer pH 5.5 at 30°C. One molar glucose monohydrate (cerelose, Corn Products Corporation) is added and completely dissolved. A crude enzyme preparation from Aspergillus niger containing glucose oxidase, lactonase, and catalase (DEE-O 1500 poweder F-5006-E, Miles Laboratories Inc.) is added to the buffered glucose solution. The impeller speed and aeration rate are then adjusted to the desired level.

The rate of production of gluconic acid by this enzyme system is linearly proportional to the oxygen uptake rate as long as the concentration of enzyme is not rate limiting and the dissolved oxygen concentration is low (10 percent of air saturation). The production of gluconic acid is followed in the 25 liter fermenter by use of a pH controller (0.1 unit dead band) set at pH 5.5. The rate of consumption of 4 N NaOH by the pH stat at pH 5.5 is equal to the rate of

FIGURE 11



$$2\ \text{Glucose} + O_2 \xrightarrow[\text{LACT.}]{\text{G.O.}} 2\ \text{Gluconic Acid}$$

CAT.

acid production. The oxygen transfer rate of the fermenter under the specified conditions of aeration rate and agitation is obtained by multiplying the rate of gluconic acid production by 0.5.

The advantages of this method over chemical oxidation are that this system has rheological properties similar to those of fermentation broth and that the oxygen transfer rate is determined by a simple automated titration.

N. Calculations

1. Moles of Oxygen Used During Oxygen-Enrichment

The moles of oxygen used during oxygen-enrichment was determined from the difference in the compressed oxygen cylinder pressure assuming a value of 1.5 $ft.^3$ for the volume of the cylinder.

$$\Delta n = \frac{V}{RT} \Delta P \qquad \text{(xi)}$$

where n = moles of oxygen

V = volume of cylinder

P = pressure in psia

T = temperature in °K

R = gas constant

Sample Calculation:

$T = 29°C = 302°K$

$\Delta P = 200$ psig

$$R = \frac{(14.7 \text{ psia})(359 \text{ ft.}^3/\text{lb. mole})}{(1 \text{ lb. mole})(273°K)} = 19.3 \frac{(\text{psi})(\text{ft.}^3)}{(K°)(\text{lb. mole})}$$

$$\Delta n = \frac{(1.5 \text{ ft.}^3)(200)}{(19.3)(302)} = 5.14 \times 10^{-2} \text{ lb. mole } O_2$$

$$n = (5.14 \times 10^{-2} \text{ lb. mole } O_2) \frac{(32 \text{ lb. } O_2)}{\text{lb. mole}} \frac{(454 \text{ gms.})}{\text{lb.}} \frac{(1 \text{ gm. mole } O_2)}{32 \text{ gm. } O_2}$$

$n = 23.3$ moles O_2 used

2. Oxygen Uptake Rate

The rate of change of dissolved oxygen in a microbial culture can be described as:

$$\frac{dc}{dt} = \text{oxygen transfer rate} - \text{oxygen uptake rate} \qquad \text{(xii)}$$

During the period of oxygen-enriched aeration the dissolved oxygen tention was maintained at a constant level, $\frac{dc}{dt} = 0$. Therefore:

oxygen transfer rate = oxygen uptake rate

The oxygen uptake rate can be calculated from the inlet and effluent oxygen concentration by the following:

Sample calculation:

P_{O_2} in = 0.21 atm.

P_{O_2} out = 0.20 atm.

Pressure = 14.7 + 2 psig

Temperature = 29°C = 302°K

Aeration Rate = 810 l/hr.

$$(810 \text{ l/hr.}) \frac{(16.7 \text{ psia})}{(14.7 \text{ psia})} \frac{(273^\circ\text{K})}{(302^\circ\text{K})} = 837 \text{ liters of gas/hr.}$$

$\Delta P_{O_2} = 0.01$ atm.

$$\text{liters } O_2\text{/hr.} = (837)(0.01) = 8.37 \text{ L. } O_2\text{/hr.}$$

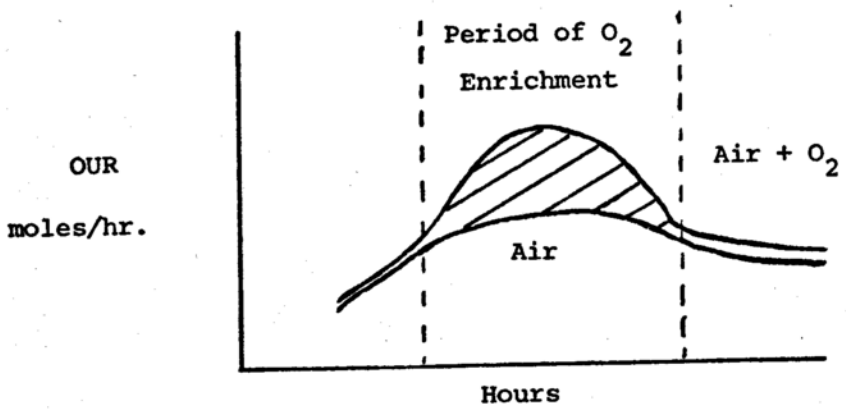
$$\frac{8.37 \text{ L. } O_2\text{/hr.}}{22.4 \text{ L. } O_2\text{/mole}} = 0.373 \text{ moles } O_2\text{/hr.}$$

3. Amount of Enriched Oxygen Used by the Culture

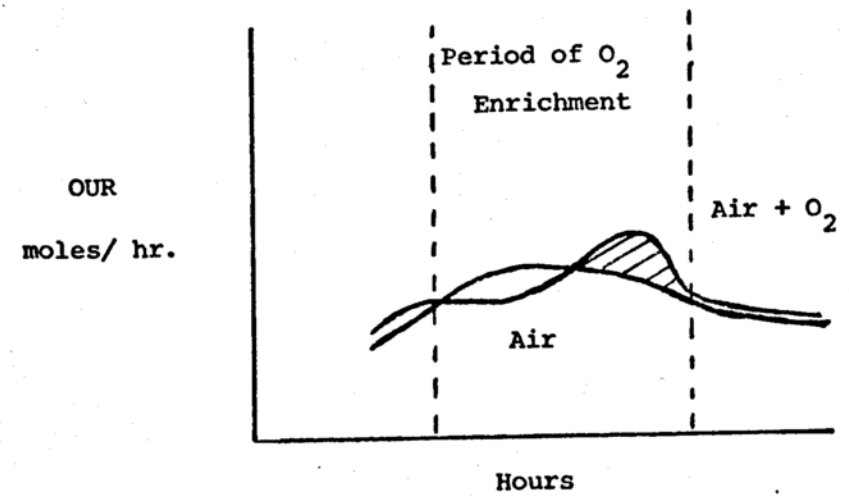
The oxygen uptake rate calculated from the inlet and effluent oxygen concentration data is plotted for each hour of the fermentation

on a linear plot against time. The oxygen uptake rate values for an identical fermentation without oxygen-enrichment are then plotted on the same graph over the corresponding time period. The area between these two curves represents the moles of oxygen enrichment actually taken up by the culture. If the oxygen uptake rate values for the oxygen-enriched fermentation are not greater than the oxygen uptake rate values for the fermentation using air, only those regions where the oxygen-enriched values are above the unenriched can be used for this calculation. This area is determined by cutting out and weighing a photocopy of the graph.

Sample plot:



or:



This calculation assumes:

1. That the physiological conditions of the cells is not significantly different in the two fermentations during the period of time where oxygen-enrichment is being used.
2. That the oxygen transfer characteristics of the broth do not change drastically during the period of oxygen-enrichment and that these characteristics are similar during both fermentations being compared.
3. That the dissolved oxygen tension during the fermentation without oxygen-enrichment is approximately zero during the time period corresponding to the period of oxygen-enrichment.

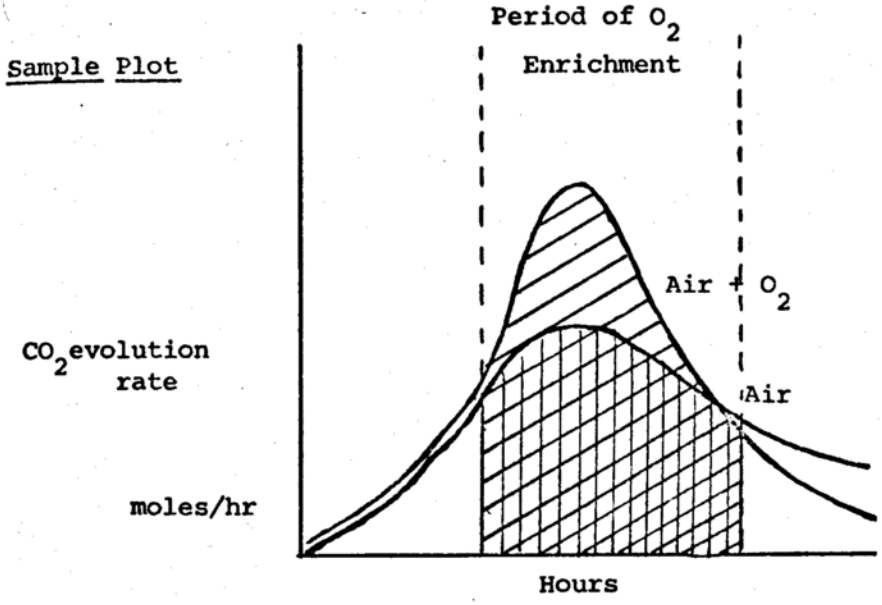
4. Carbon Dioxide Evolution Rate

The gas phase carbon dioxide evolution rate was calculated from the inlet and effluent pCO_2 assuming that the concentration of CO_2 in the inlet air is 0.0025 atm. The calculation is identical to that for determination of the oxygen uptake rate (section N - 2).

The total moles of CO_2 produced during a fermentation was determined from the area (weight method) under a linear plot of the CO_2 evolution rate data (moles/hr.) against time in hours.

The relative CO_2 production during the period of oxygen-enrichment was determined from the area under the curves for the CO_2 evolution rate for identical fermentations with and without oxygen-enrichment over the period of time corresponding to that of oxygen-enrichment.

Sample Plot



5. Respiratory Quotient

The respiratory quotient wascalculated for each hour of the fermentation from the quotient of the carbon dioxide evolution rate and the oxygen uptake rate and is dimensionless.

6. Specific Growth Rate

The specific growth rate, μ, was determined from the following formula:

$$\mu = \frac{\ln N_2 - \ln N_1}{(t_2 - t_1)} \text{ (Hr.}^{-1}\text{)} \qquad \text{(xiii)}$$

where N_2 = cell mass at time t_2

N_1 = cell mass at time t_1

7. Correction of Bioactivity Data for Differential Response of Neomycin B and C

The total grams of neomycins produced during S. fradiae fermentations was determined by B. subtilis bioassay against neomycin

standard (lot. no. 53-NHF-1) containing less than five percent neomycin C (determined by GLC method). Since the major component neomycin B is more active than C, bioassay results for total neomycins will give low values if they contain more neomycin C than the five percent present in the standard. The relative antimicrobial activity of neomycin B to C also varies with the species of test organism used (61,62).

A relationship, modified from the method of Tsuji (62), for correction of the total weight of neomycins present was used in fermentations containing more than five percent neomycin C. This method determines the total weight of neomycin present from the total antimicrobial activity, ratio of neomycin B to C (determined by GLC method), and the relative biological response of neomycin B and C for the test organism B. subtilis (Marburg).

$$B_f \times B_r + C_f \times C_r = \text{total biological activity} \qquad \text{(xiv)}$$

where B_f = (total weight)(percent neomycin B)

C_f = (total weight)(percent neomycin C)

B_r, C_r = relative biological response of neomycin B and neomycin C respectively

If the total biological activity and the precentage of neomycin C present are determined, the weight of total neomycins can be calculated by rearrangement of equation (xiv):

$$\text{total weight of neomycins} = \frac{\text{total biological activity}}{(\%\ \text{neo. C})(C_r) + B_r - (\%\ \text{neo.C})(B_r)}$$

V. RESULTS

A. Oxygen Transfer Rate of the Fermenter

1. Comparison of the Two Methods

The Oxygen Transfer Rate (OTR) of the fermenter was determined for distilled water at 30°C using both the Sulfite Oxidation (p. 58 and Glucose Oxidase methods (p. 60). Table II compares the OTR determinations by these two methods. The enzymatic determinations were performed one year after the Sulfite Oxidation method. Several reasons may exist why the enzymatically determined values are lower than the sulfite oxidation values:

1) the impeller tachometer no longer represented the true rpm due to stretching of the belt drive over one year of operation.
2) the enzymatic determination foamed excessively which may have inactivated the protein.
3) the rheology of the enzymatic determination (1 molar glucose + 4 grams/l protein) may more accurately reflect actual fermentation conditions suggesting that the OTR actually was significantly lower than when determined in a salts solution.

Even though 4 grams/liter of the glucose oxidase-lactonase-catalase preparation was not completely soluble, the OTR values do not indicate that enzyme concentration was limiting at 400 rpm with 1.0 vvm (volumes of air per volume of medium/minute) aeration rate. The enzymatic method employed (60) was reported for a 1.5 liter fermenter. No mention was made of application to larger scale volumes.

Table II

DETERMINATION OF THE OXIDATION TRANSFER RATE OF THE FERMENTER AT 30°C

Impeller Speed RPM	Aeration Rate vvm[1]	Sulfite Oxidation Method	Glucose Oxidase Method
100	0.05	n.d.[2]	0.03 mM/l./min.
200	0.5	n.d.	0.13
300	0.5	n.d	0.38
400	0.5	n.d.	0.64
100	1.0	0.15	n.d.
200	1.0	0.40	0.12
300	1.0	0.62	0.33
400	1.0	0.80	0.71
400	1.5	1.7	n.d.

[1] vvm = volumes of air per volume of medium per minute

[2] n.d. = not determined

2. Physical Factors Effecting Oxygen Transfer

a. Impeller Speed and Aeration Rate

The date in table II confirms that impeller speed is more effective in changing the rate of oxygen transfer than is manipulation of the aeration rate. The OTR determined by sulfite oxidation suggests that oxygen transfer can be increased five fold by increasing the impeller speed from 100 to 400 rpm. In contrast, the OTR values determined enzymatically at both 1.0 vvm and 0.5 vvm are essentially identical at each impeller speed examined.

b. Antifoam

Table III summarizes the effect of the addition of silicone antifoams on the rate of oxygen transfer. Addition of 120 ppm (0.12 ml/liter) of SAG 4130 and 500 ppm of SAG 5440 antifoam reduced the OTR by 34 percent and 29 percent respectively. This data suggests that control of frothing by addition of these silicone antifoams reduced the OTR during the fermentation by one third. The data also show that addition of small quantities of surface active antifoams (5 ppm) sharply reduced the OTR which gradually increased with increasing additions (120 ppm). This previously investigated phenomenon has been attributed to the decreasing air bubble size with the addition of surface active agents (63).

c. Glycerol

Table III also shows the effect of the addition of glycerol. Concentration of 15-100 gms/l decreased the OTR by 8 to 15 percent.

In the fermentation media the addition of complex organic substances (63), antifoams, and even glycerol can reduce oxygen transfer so that it could become a limiting factor.

Table III

THE EFFECT OF ADDED ANTIFOAM AND GLYCEROL ON THE OXYGEN TRANSFER RATE

Antifoam

Antifoam	ml./l/	Method	Impeller RPM	Aeration Rate	OTR[1]	Relative OTR
None	-	S.O.[4]	400	1.5 vvm	1.7	1.0
4130[2]	0.005	S.O.	400	1.5	1.0	0.59
4130	0.02	S.O.	400	1.5	0.94	0.55
4130	0.04	S.O.	400	1.5	0.97	0.57
4130	0.12	S.O.	400	1.5	1.1	0.66
None	-	G.O.[5]	300	1.0	0.33	1.0
5440[3]	0.05	G.O.	300	1.0	0.21	0.63
5440	0.50	G.O.	300	1.0	0.23	0.71

Glycerol

Glycerol	Method	Impeller RPM	Aeration Rate	OTR[1]	Relative OTR
None	S.O.	400	1.5	1.7	1.0
15 gm/1.	S.O.	400	1.5	1.5	0.85
30	S.O.	400	1.5	1.6	0.91
100	S.O.	400	1.5	1.6	0.91

(1) Oxygen Transfer Rate in millimoles oxygen per liter per minute
(2) Union Carbide SAG 4130 silicone antifoam
(3) Union Carbide SAG 5440 silicone antifoam
(4) S.O. - Sulfite Oxidation
(5) G.O. - Glucose Oxidase

B. Conversion of Glycerol to Dihydroxyacetone by Glyconobacter melanogenus IFO 3293

1. Industrial Process

The current industrial production of 1,3 dihydroxyacetone (DHA) involves the bio-conversion of glycerol to DHA by strains of Acetobacter suboxydans (64,65,66,67,68,69,70). These strains are grown on a medium containing: glycerol, 100-120 gms./liter, cornsteep liquor, 5 gm./liter, Brewer's yeast, 5 gm./liter, KH_2PO_4, 5 gm./liter, and calcium carbonate, 20 gms./liter. The pH optimum for this conversion is pH 5.5 with reported yields of 95 percent in 24 to 36 hours (70).

The major industrial uses of DHA include cosmetic formulations which produce tanning of the epidermal layers of the skin simulating a suntan and as a catalyst in industrial polymerization reactions. The types of biological activity attributed to DHA include acting as a respiratory stimulant, in the treatment of diabetes and hypoglycemia, and some antiviral activity (69). Owing to its carbonyl group and two primary alcohol groups, DHA is capable of taking part in many reactions including autocondensation and polymerization (71).

During the investigation of the converstion of L-sorbose to L-sorbosone by Gluconobacter melanogenus IFO 3293 (72,73,74,75) it was noted that large quantities of dihydroxyacetone(DHA) accumulated in the medium when the organism was grown in a medium containing both glycerol and L-sorbose. The quantity of DHA accumulated appeared to be directly related to the amount of oxygen supplied to the fermentation. This system was therefore chosen for investigation of the possible utility of applying oxygen enriched aeration for extending the oxygen transfer capability of the vessel in which the organism was grown.

2. Pathway of Glycerol Metabolism in Acetobacter Species

The pathway of glycerol metabolism in acetic acid bacteria has been studied in both whole cell and cell free preparations. Figure 12 describes the oxidative pathway of glycerol and the pentose cycle in A. suboxydans after the studies of King and Cheldelin (76,77,78, 79,80,81) The oxidation of glycerol proceeds by two paths: One is apparently independent of ATP and NAD and proceeds optimally at pH 6 to the formation of dihydroxyacetone; the second, with a pH optimum of 8.5 requires the participation of ATP and Mg^{++} in a kinase reaction to yield glycerol -α-phosphate. The latter is then oxidized by a NAD-dependent dehydrogenase to form dihydroxyacetone phosphate.

A similar path for glycerol metabolism has been implied for Gluconobacter species by Asai (82). In Gluconobacter suboxydans, the pentose cycle is overshadowed during growth by the nonphosphorylative oxidation which accumulates DHA more rapidly that it can be metabolized further. (82)

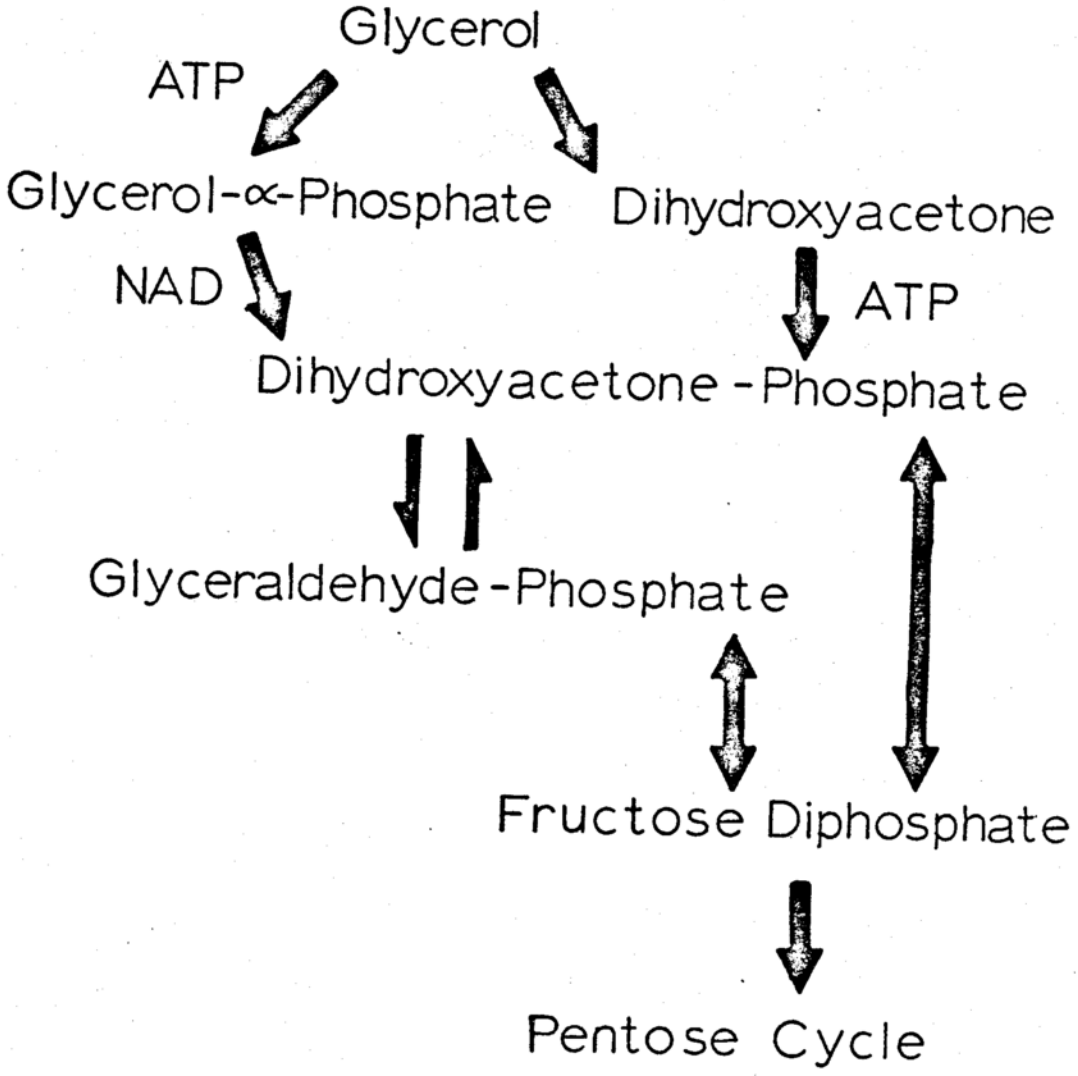
The current industrial process for DHA formation relies on maintenance of a slightly acid environment (pH 5.5) which may operate to prevent functioning of both glycerol and DHA kinases and effectively inhibit further utilization of DHA by producing strains of A. suboxydans (70).

3. Nutritional Studies

a. Growth on a Defined Medium

Attempts were made to define the nutritional requirements of G. melanogenus 3293 in order to study the conversion of glycerol to DHA in a defined medium. The organism was capable of growth on a defined medium reported for the growth of Acetobacter melanogenus by Foda and

FIGURE 12


Glycerol
ATP
Glycerol-α-Phosphate
Dihydroxyacetone
NAD
ATP
Dihydroxyacetone-Phosphate
Glyceraldehyde-Phosphate
Fructose Diphosphate
Pentose Cycle

Vaughn (83) (Table IV). Twenty millimolar pH 7 phosphate buffer was added to this medium to maintain the pH at 7 throughout the 48 hours of incubation. Carbon sources were added at the level of 10 gm./liter. All vitamins were filter sterilized through a 0.20 micron membrane filter (Nalgene) and aseptically added after sterilization of the medium.

The results of the nutritional study (Table V) suggest: 1) glucose can be utilized as a sole source of carbon and energy although glycerol, casein hydroyzate (Difco), N-Z amines (Humko Sheffield) or glutamate cannot; 2) the cell yield of the Foda and Vaughn medium is not limited either by the concentration of any one vitamin or trace metals; 3) pantothenic acid is required for growth of the culture in the presence of glucose, PABA and nicotinic acid are not required but stimulate growth, and thiamine is neither required not stimulatory for growth; 4) ammonium sulfate is preferred as a source of cell nitrogen over glutamate, casein hydroyzate, or N-Z amines. Casein hydroyzate can be utilized as a source of nitrogen in the presence of glucose but not in the presence of glycerol; 5) growth of the culture on the defined medium plus glycerol is greatly stimulated by the presence of yeast extract. The cell yield on the Foda and Vaughn medium in the presence of 10 gm./liter of glycerol and 5 gm./liter yeast extract is over six fold that when glucose alone is utilized.

Glycerol cannot be utilized, presumably initially as an energy source, without some unknown factor present in the yeast extract. This factor has been demonstrated not to be any water soluble vitamin, vitamin B_{12}, inositol, adenosine or any single amino acid. No further characterization of this factor has been done. A similar finding has

Table IV DEFINED MEDIUM FOR GROWTH OF A. melanogenus

After Foda and Vaughn (83)

	grams per liter
KH_2PO_4	0.5
$K_2HPO_4 \cdot 3H_2O$	0.5
$MgSO_4 \cdot 7H_2O$	0.2
$FeSO_4 \cdot 7H_2O$	0.01
$MnSO_4 \cdot 4H_2O$	0.01
NaCl	0.01
$(NH_4)_2SO_4$	10.0
Pantothenic Acid	1000 micro grams
PABA	200 micro grams
Nicotinic Acid	200 micro grams
Thiamine - HCl	100 micro grams
Glucose	10 grams
Distilled water	1000 ml.

Table V GROWTH OF G. melanogenus 3293 ON VARIOUS DEFINED MEDIA

Additions to basal medium[1]	Cell yield in gms dry wt./l.[2]
none	0.00
glucose	0.18
glycerol	0.00
casein hydroyzate (vitamin free)	0.00
N-Z amine type A or B	0.00
casein hydrolyzate + glucose	0.13
casein hydrolyzate + glycerol	0.00
glutamate + glycerol	0.00
N-Z amine type A or B + glycerol	0.00
glycerol + yeast extract	2.88
2 x vitamin components + glucose	0.12
2 x trace metal components + glucose	0.18
glucose - pantothenic	0.00
glucose -PABA	0.09
glucose - nicotinic acid	0.12
glucose - thiamine	0.18
glucose - $(NH_4)_2SO_4$	0.00
casein hydrolyzate - $(NH_4)_2SO_4$	0.00
casein hydrolyzate + glucose - $(NH_4)_2SO_4$	0.16
glutamate - $(NH_4)_2SO_4$	0.00
N-Z amine type A or B - $(NH_4)_2SO_4$	0.00
casein hydrolyzate + glycerol - $(NH_4)_2SO_4$	0.01

[1] Basal medium - medium in Table IV without glucose
[2] Calculated from change in absorbance at 650 nm after 48 hours incubation at 30°C - details in text

been reported in the study of other Gluconobacter species on defined media (84,85).

Unfortunately, glycerol is not quantitatively converted to DHA in the presence of glucose when the organism is grown on the defined medium of Foda and Vaughn. This has limited the study of the effect of oxygen on this bio-conversion to yeast extract containing medium.

b. Effect of Niacin on Conversion

Growth of the organism in a glycerol-yeast extract medium containing more than five grams per liter of yeast extract (Amber 1003) results in inhibition of conversion in 25 liter fermentations. Fermentation media containing more than 5 gms./liter of yeast extract produced more cell mass with reduced accumulation of DHA. This data is shown in figure 13 and table VI. A nutritional study of the nature of this inhibition in 250 ml. Erlenmeyer shake flasks revealed that the addition of niacin to the yeast extract gave the same response. Twenty milligrams per liter of niacin added to the glyverol-yeast extract fermentation medium can inhibit total molar conversion by 89 percent in the presence of air and by 36 percent when using oxygen-enriched aeration.

c. Apparent Dihydroxyacetone Toxicity to G. melanogenus

During nutritional studies on G. melanogenus 3293 it was observed that concentrations of DHA greater than 2 gms/liter inhibited all cell growth when added to shaken cultures of the inoculum medium (400 ml. per 2000 ml. Erlenmeyer flask, 250 rpm rotary shaker, 30°C) at the time of inoculation. (Figure 14). Addition of from 0.2 to 1.5 gms./ liter of DHA at the time of inoculation increased the lag time of the culture from 8 to 12 hours (table VII). However, neither the growth rate nor the final cell yield of the culture were affected by addition

FIGURE 13

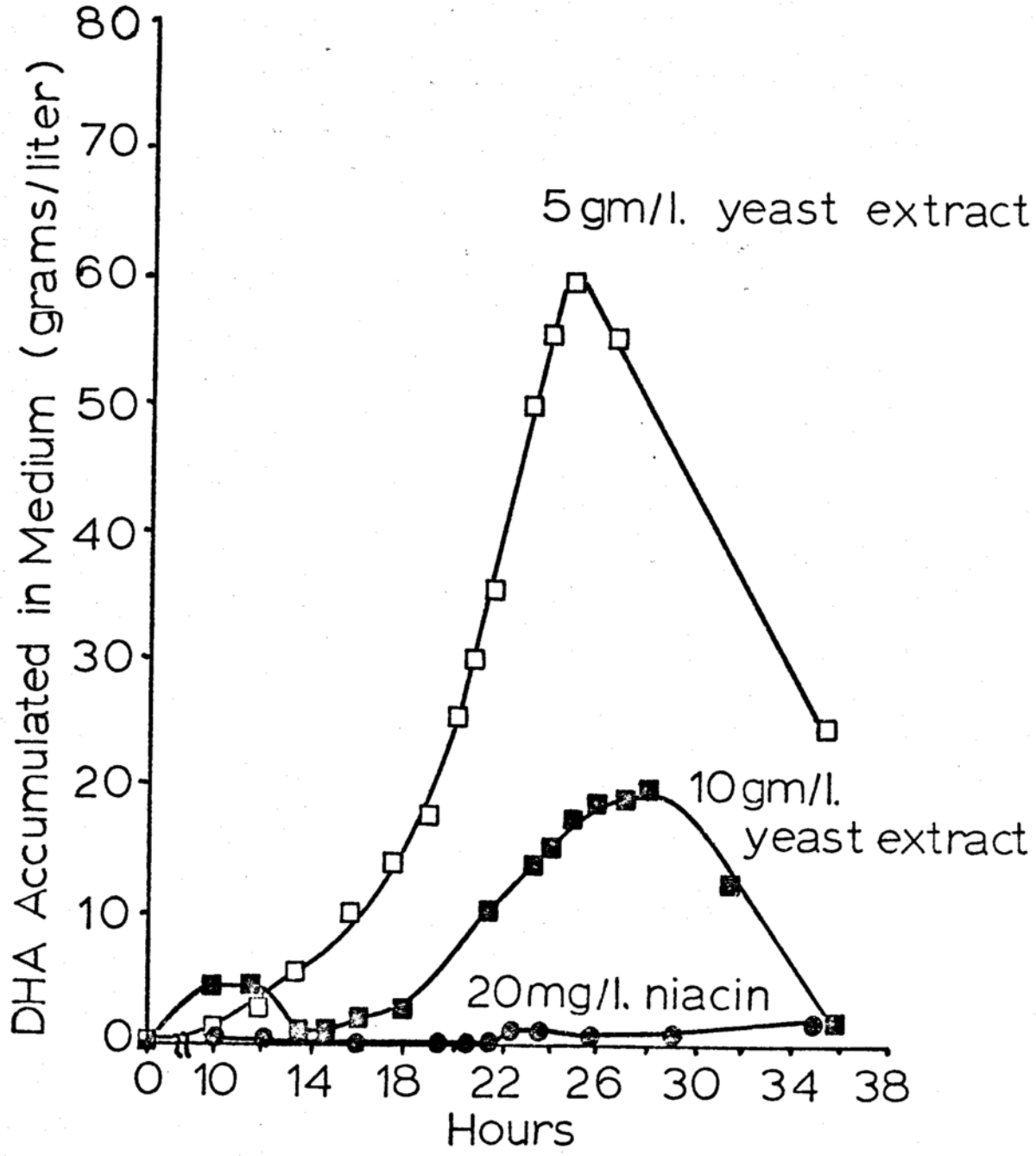

DHA Accumulated in Medium (grams/liter)
80
70
60
50
40
30
20
10
0
5 gm/l. yeast extract
10 gm/l.
yeast extract
20 mg/l. niacin
0 10 14 18 22 26 30 34 38
Hours

Table VI

EFFECT OF ADDED NIACIN ON CONVERSION OF GLYCEROL TO DIHYDROXYACETONE BY *G. melanogenus*

APPROXIMATE INITIAL GLYCEROL CONCENTRATION	AERATION AT 1.5 VVM	MAXIMUM DHA ACCUMULATED	CELL MASS AT TIME OF MAX. CONVERSION	GM. DHA PER GM. CELL MASS AT MAX. CONVERSION
100 gm/l	no oxygen enrichment	36.0 gm/ l	2.95 gm/l	12.2
100 (20 mg/l Niacin)	no oxygen enrichment	4.0	7.25	0.55
100	oxygen enrichment	102.0	2.95	35.0
100 (20 mg/l Niacin)	oxygen enrichment	36.0	7.25	5.0

CONDITIONS: 400 rpm, 30°C, pH 7.0
oxygen enrichment to maintain 0.05 atm. dissolved oxygen

FIGURE 14

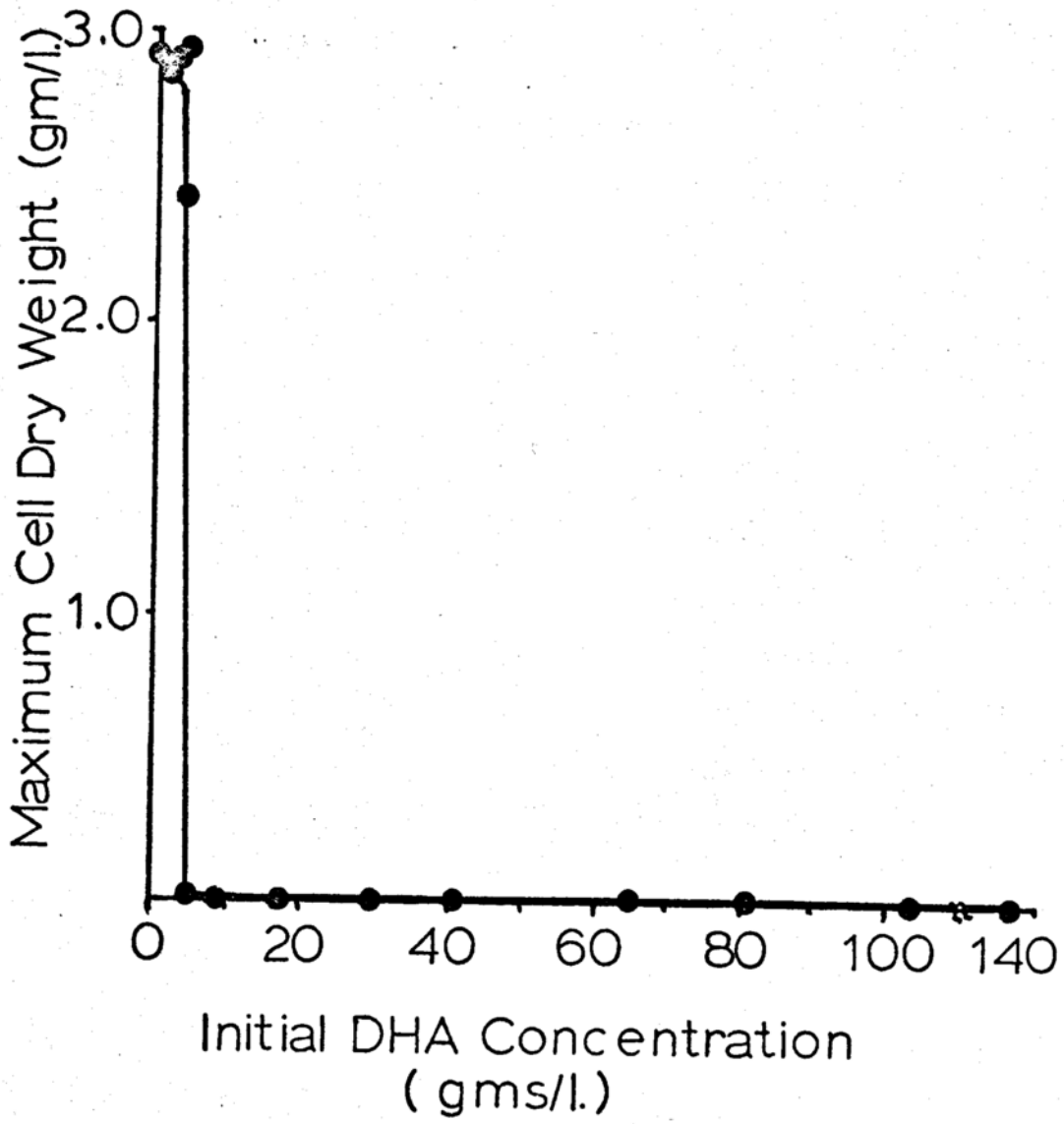

Maximum Cell Dry Weight (gm/l.)
3.0
2.0
1.0
0
20
40
60
80
100
140
Initial DHA Concentration
(gms/l.)

Table VII

EFFECT OF DIHYDROXYACETONE PRESENT INITIALLY ON LENGTH OF LAG TIME OF *G. melanogenus*

Initial Concentration of Dihydroxyacetone in grams/liter	Lag Time in Hours
0	8.2
0.2	9.2
0.5	11.0
1.0	11.4
1.5	12.1
2.0	No Growth
3.0	No Growth
4.0	No Growth
5.0	No Growth

Conditions: shake flask medium (page 27)

400 ml. per 2000 ml. flask

30°C

pH 7

15 grams per liter glycerol initially

250 rpm shaker

of less than 1.5 gms./liter of DHA at the time of inoculation. This apparent toxicity of low concentration of DHA to non-dividing or slowly dividing cells of G. melanogenus 3293 may explain the necessity of maintaining the culture by transferring it to fresh slants every two weeks. Viability of the cells on agar slants decreased substantially after two weeks when stores at 4°C.

4. Growth Associated Accumulation of DHA in 25 liter Fermentations

The conversion of 15 gms./liter of glycerol to dihydroxyacetone (DHA) is shown in figure 15. The rate of DHA accumulation is the same as the rate of cell mass increase demonstrating that the conversion occurs associated with expoential cell growth. Fifteen gms./liter of glycerol is quantitatively converted to DHA in 12 hours followed by the apparent further metabolism of DHA. Accumulation of DHA stops with exhaustion of glycerol from the medium.

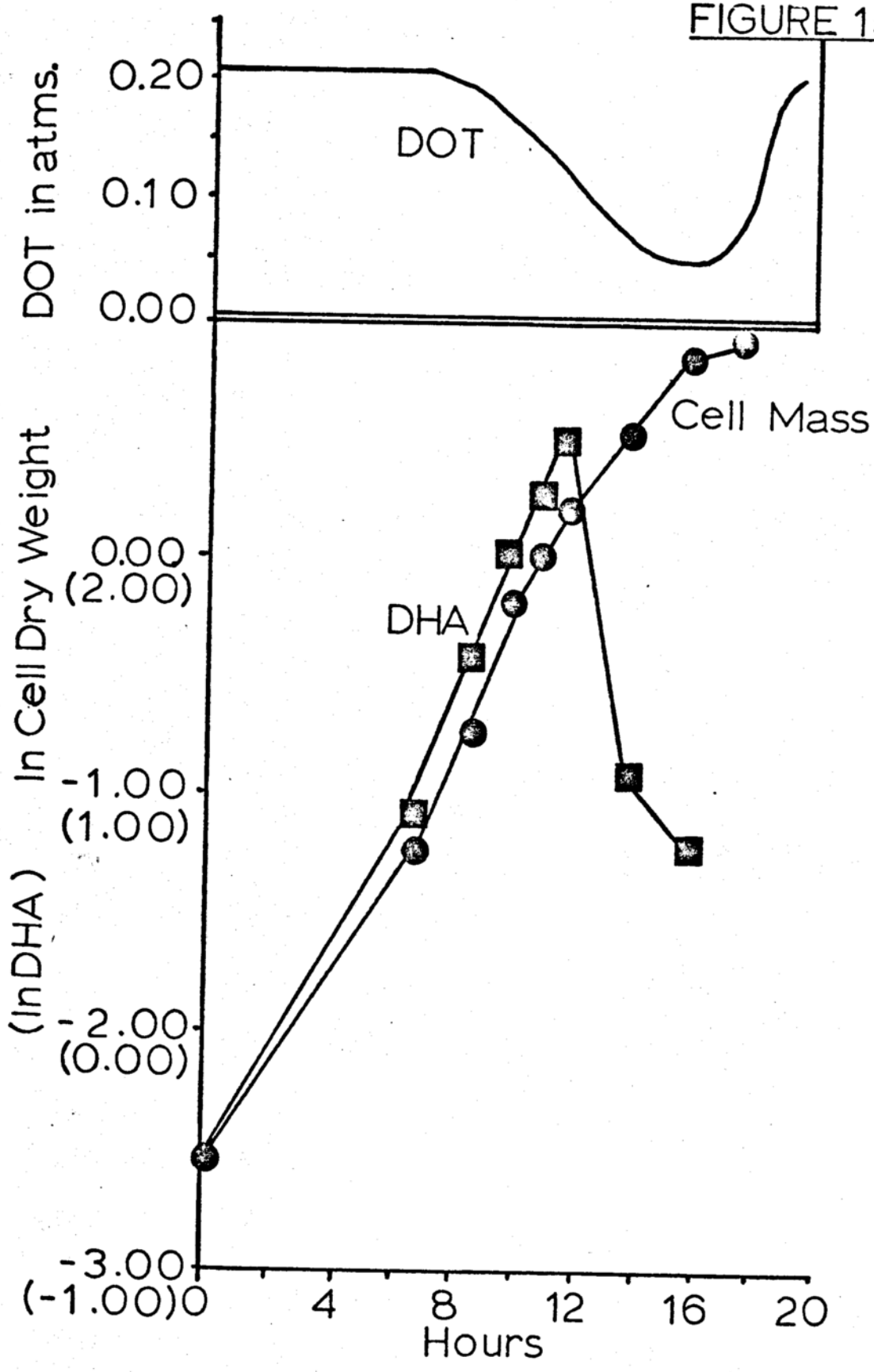
It is also noted by recording the rate of consumption of NaOH by pH controller that acid products accumulate with increasing cell mass and that this rate does not change after the point where DHA accumulation ceases. Also shown in figure 15 is the behavior of the dissolved oxygen during conversion of 15 gms./liter of glycerol to DHA. Under the conditions of 400 rpm impeller speed with 1.5 vvm aeration rate sufficient oxygen is transferred to satisfy the oxygen demand of the fermentation without the culture becoming oxygen limited.

5. Method for Inhibition of Further DHA Metabolism

Bio-conversion of glycerol to DHA reaches a peak of maximum accumulation in the medium followed by further metabolism of the dihydroxyacetone. In order to stop this bio-conversion at the point of quantitative conversion, further metabolism must be blocked.

The metabolic uncoupler, 2,4 dinitrophenol (DNP), has been

FIGURE 15


DOT in atms.
0.20
0.10
0.00
DOT
Cell Mass
DHA
ln Cell Dry Weight
0.00
(2.00)
-1.00
(1.00)
(ln DHA)
-2.00
(0.00)
-3.00
(-1.00)0
4
8
12
16
20
Hours

reported by King and Cheldelin to inhibit further DHA metabolism by uncoupling the DHA kinase reaction (78). Their cell-free study also demonstrated that conversion of glycerol to DHA was not affected by addition of DNP.

Shaken flask studies utilizing 200 ml. of the fermentation medium in 2000 ml. Erlenmeyer flasks on a 300 rpm rotary shaker at 30°C demonstrated that DNP could be used to inhibit further DHA metabolism in G. melanogenus. In this study an acetone solution of DNP was added to 15-hour cultures containing 15 grams per liter of glycerol initially. A level of 2×10^{-2} molar DNP was required to inhibit DHA metabolism in these rapidly growing cells. This level is too high to be of practical use for a large scale process.

If the temperature of a 25 liter batch fermentation of G. melanogenus growing on the glycerol-yeast extract medium is abruptly dropped from 30°C to 10°C in 15 minutes by the addition of ice and all aeration is shut off, 25 to 30 percent of the accumulated DHA is metabolised in 20 hours at 10°C (Figure 16).

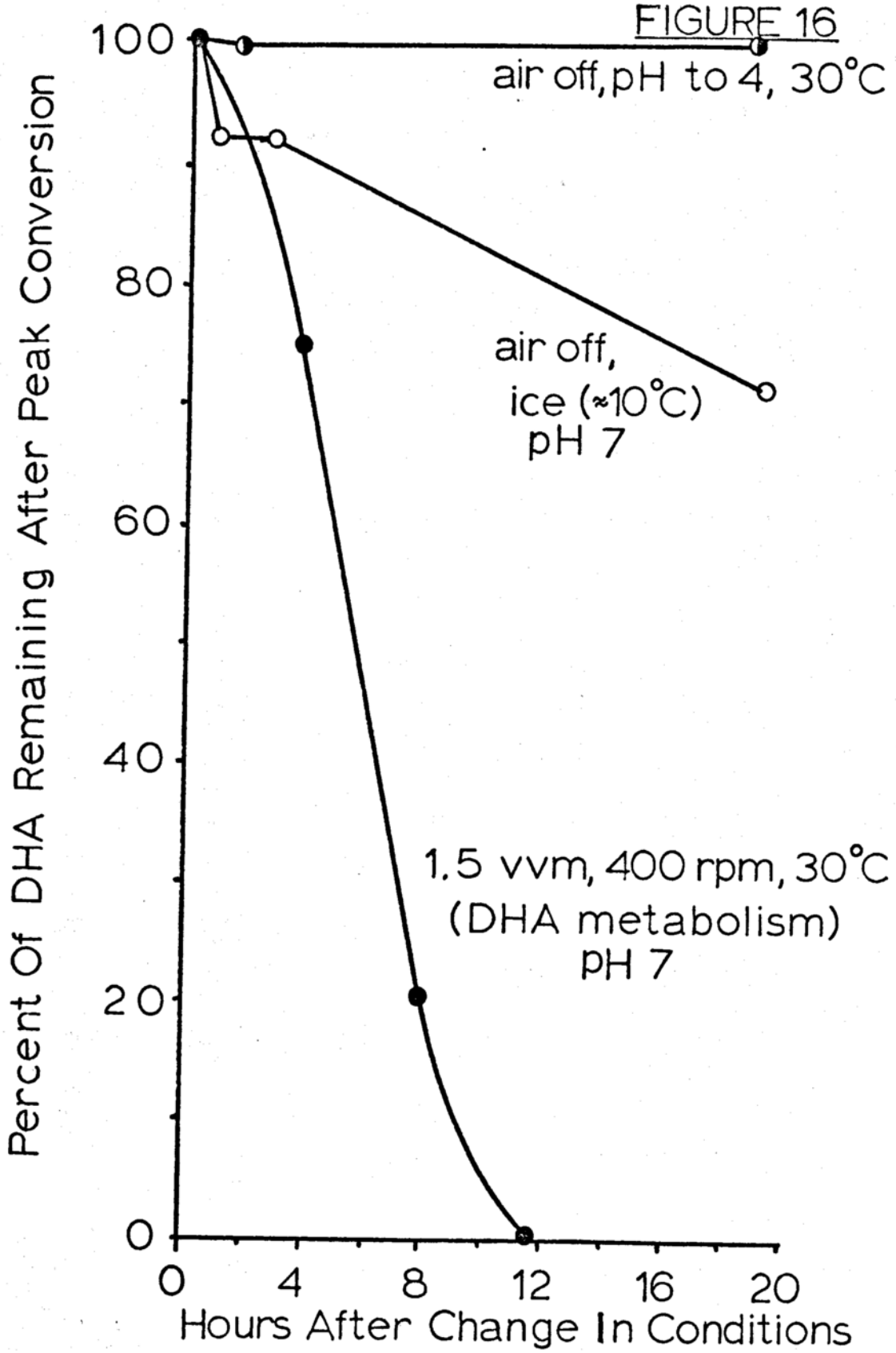
The most effective method for inhibition of further DHA metabolism is by acidifying the fermentation to pH 4 with 2 N H_2SO_4. The fermentation beer can be stored at this low pH without aeration at 30°C for 20 hours without further metabolism of accumulated DHA. (Figure 16) Both the temperature drop and the pH drop methods inhibit conversion of glycerol to DHA and therefore must be made as close to the time of peak accumulation as possible.

6. The Effect of Oxygen on Conversion

a. Oxygen Limitation

When the initial glycerol concentration was 30 gms./liter, fermentation became oxygen limited shortly before complete

FIGURE 16


air off, pH to 4, 30°C
air off, ice (≈10°C) pH 7
1.5 vvm, 400 rpm, 30°C (DHA metabolism) pH 7
Percent Of DHA Remaining After Peak Conversion
100
80
60
40
20
0
0
4
8
12
16
20
Hours After Change In Conditions

conversion was attained. The effect of oxygen limitation on conversion was even more evident when the initial glycerol level of the fermentation was increased to 50 gms./liter. Data summarized in figure 17 shows that conversion of glycerol to DHA appears to cease at the point where the culture becomes oxygen limited. In this case, the accumulation of DHA is only 59 percent of the original glycerol concentration (on a molar basis). During this period of oxygen limitation (dissolved oxygen approximately zero) the accumulated DHA is not rapidly metabolized as was observed where sufficient oxygen was available (figure 15). The accumulation of acid products continues to increase during this period of oxygen limitation.

b. Oxygen-Enriched Aeration

The problem of oxygen limitation causing conversion to stop could be overcome by simply increasing the aeration rate. However, when this procedure was tried using an initial glycerol concentration of 30 gms./liter, the aeration rate at constant impeller speed required to allow for quantitative accumulation of DHA was found to be 3.5 vvm.

An alternative method for satisfying this high oxygen demand without changing the aeration rate or impeller speed is through oxygen enrichment (figure 18). This fermentation was run as described in figure 17, however, as the oxygen tension decreased below 0.05 atm., the aeration was enriched with compressed oxygen at a constant total aeration rate. Thus, this fermentation never became oxygen limited. As the oxygen demand of the culture decreased, the oxygen enrichment was decreased so as not to exceed 0.05 atm. of dissolved oxygen. Seven hours of oxygen enrichment was required

FIGURE 17

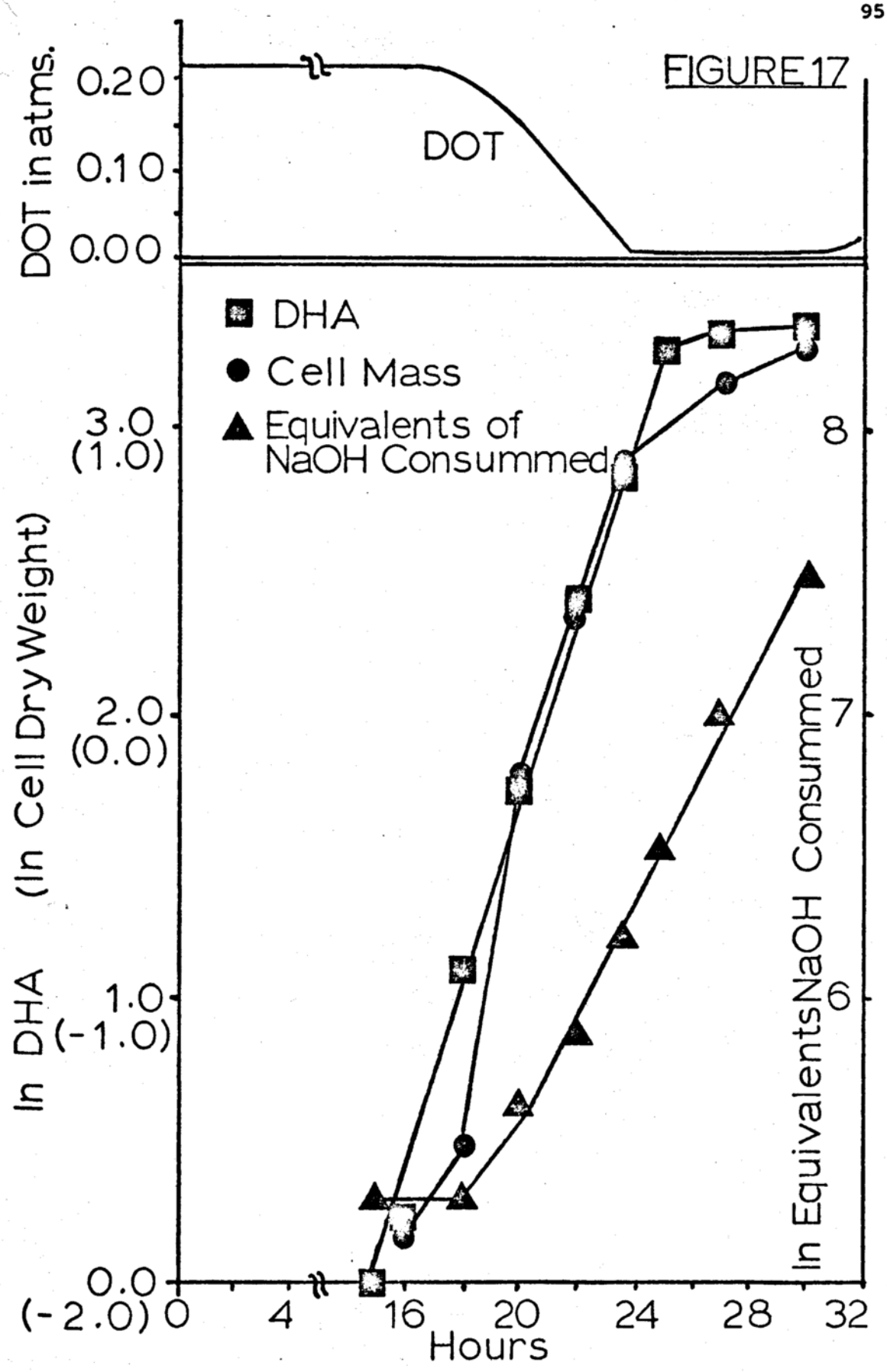
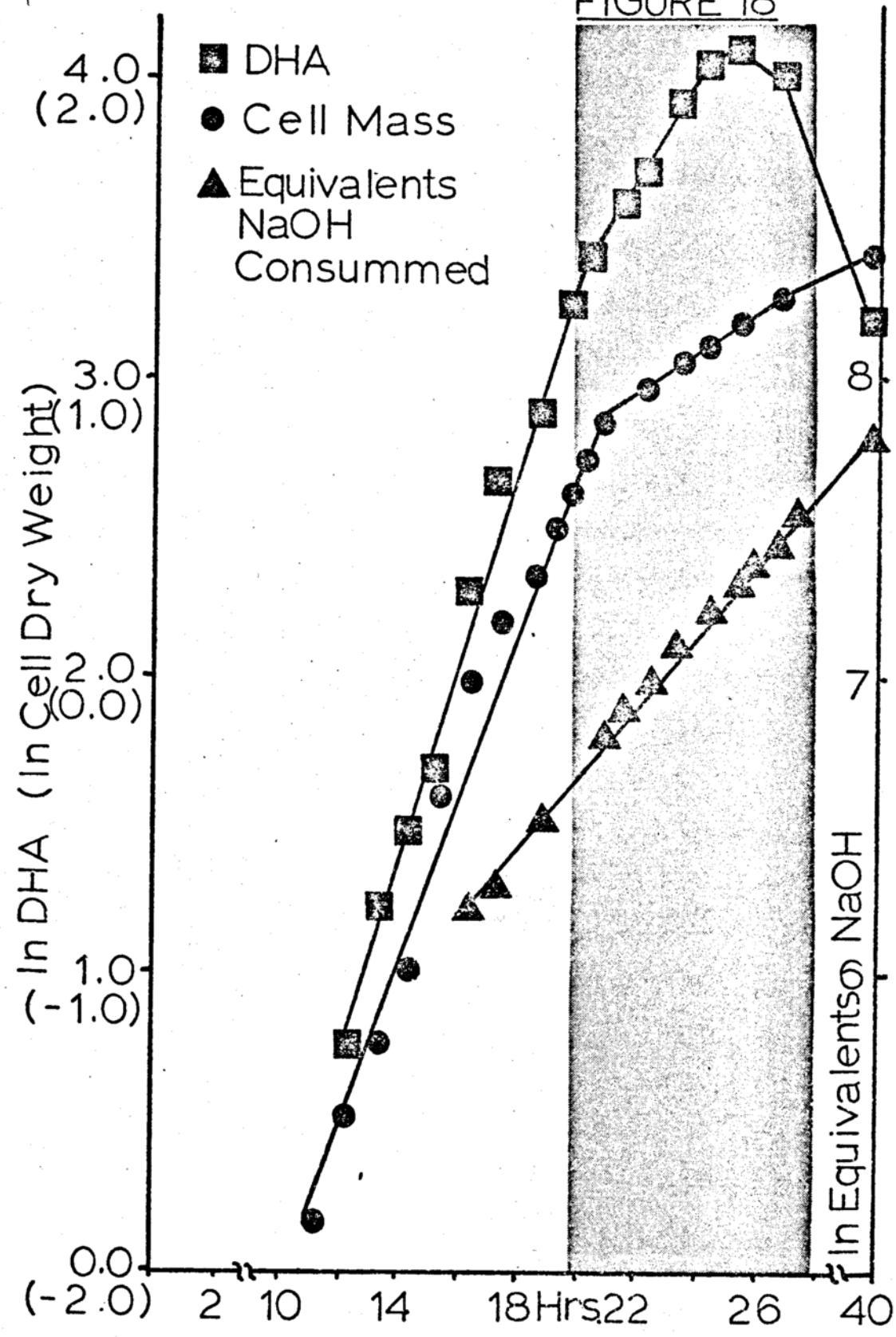

DOT in atms.
0.20
0.10
0.00
DOT
DHA
Cell Mass
Equivalents of NaOH Consummed
3.0
(1.0)
2.0
(0.0)
1.0
(-1.0)
0.0
(-2.0)
ln DHA (ln Cell Dry Weight)
8
7
6
ln Equivalents NaOH Consummed
0
4
16
20
24
28
32
Hours

FIGURE 18


DHA
Cell Mass
Equivalents NaOH Consummed
4.0
(2.0)
3.0
(1.0)
2.0
(0.0)
1.0
(-1.0)
0.0
(-2.0)
(In DHA (In Cell Dry Weight)
8
7
In Equivalents NaOH
2
10
14
18 Hrs
22
26
40

to maintain a constant level of oxygen tension.

During the period of oxygen enrichment, the conversion of glycerol to DHA did not cease as seen earlier (figure 17) but continued at its original rate until quantitative conversion was attained (figure 18). Metabolism of the accumulated DHA followed. However it is also important to note that the accumulation of acid products by the culture continued at the same rate during this period of constant oxygen tension.

Of the greatest interest is the data in figure 18 showing the effect of oxygen enriched aeration on increasing cell mass. On this plot exponentially increasing cell mass is represented by a straight line, indicating that the cells continue to divide exponentially during the period of oxygen enrichment but at a much slower rate. The specific growth rate decreased at the onset of oxygen enrichment from 0.23 $hour^{-1}$ to 0.06 $hour^{-1}$.

This same effect of the ability to increase conversion with oxygen enrichment was demonstrated when 100 gms./liter glycerol served as substrate under the same conditions of impeller speed and aeration rate. When the fermenter was sparged with air, 37 percent (molar basis) of the initial glycerol was converted to DHA. However when the oxygen tension was maintained at 0.05 atm. by enrichment of the aeration with compressed oxygen, quantitative conversion (104 percent molar basis) could be attained without changing impeller speed or aeration rate. The data summarizing the effect of oxygen-enriched aeration on increasing the quantity of glycerol converted to DHA are presented in table VIII. It is interesting to note that the increase in conversion was not due to a significant increase in cell mass in the oxygen enriched fermentations.

Table VIII EFFECT OF OXYGEN-ENRICHED AERATION ON CONVERSION OF GLYCEROL TO DIHYDROXYACETONE BY G.MELANOGENUS 3293 AT VARIOUS INITIAL GLYCEROL LEVELS

Approximate Initial Glycerol Concentration	Oxygen Enrichment[1]	Molar Conversion (Percent)	Equivalents of NaOH Consumed	Cell Mass at Time of Max. Conversion	Gm. DHA per Gm. Cell Mass at Maximum
15 gm/l	None	106	370	1.27 gm/l	12.2
30	None	97	344	1.34	21.6
30	None (Increase vvm to 3.5)	106	350	1.46	22.8
30	Yes	109	560	1.45	22.3
50	None	59	1100	3.15	9.2
50	Yes	105	1460	3.32	15.4
100	None	37	640	2.95	12.2
100	Yes	104	1920	2.85	35.8

Fermentation Conditions: 1.5 vvm, 400 rpm, 30°C, pH 7.0

Oxygen Enrichment to Maintain 0.05 atm. Dissolved Oxygen

c. Relationship Between Accumulation of DHA and the Partial Pressure of Oxygen in the Sparge Gas

The effect of oxygen tension on conversion was further investigated by sparging the fermenter with a reduced pO_2 level. Since the use of increased pO_2 demonstrated increased conversion without increasing cell mass, we wished to determine whether the opposite condition could be achieved by sparging the fermenter with a gas mixture of less than the pO_2 found in air. The previous data would suggest that this would lead to decreased conversion but with the same level of cell mass production. Table IX summarizes the results of three fermentations attempting to quantitatively convert 50 gms./liter of glycerol to DHA. The first fermentation was sparged with air at 1.5 vvm. This fermentation became oxygen limited after 21 hours resulting in a molar conversion of 59 percent of the added glycerol. The fermentation was repeated with oxygen-enriched aeration to maintain 0.05 atm. dissolved oxygen with quantitative accumulation of DHA. Both fermentations resulted in production of about three grams per liter of cell dry weight. A third fermentation was sparged with a mixture of nitrogen and air sufficient to bring the volumetric oxygen content down to approximately 19 percent. With aeration using this gas mixture under the identical conditions of agitation and sparge rate, the fermentation became oxygen limited five hours earlier, resulting in accumulation of only 44 percent (molar basis) of the added glycerol as DHA. The quantity of cell mass produced by this fermentation at the time of maximum DHA accumulation was 4 gms./liter of cell dry weight, greater than either of the previous fermentations.

Table IX EFFECT OF OXYGEN CONCENTRATION IN SPARGE GAS ON DIRECTING GLYCEROL TO DIHYDROXYACETONE ACCUMULATION OR CELL MASS PRODUCTION OF G. MELANOGENUS 3293

Approximate Initial Glycerol Concentration	Aeration 1.5 vvm	Molar Conversion (Percent)	Cell Mass at Time of Max. Conversion	Gm. DHA per Gm. Cell Mass at Max. Conversion
50 gm/l	Oxygen Enrichment[1]	105	3.3 gm/l	15.5
50	No Oxygen Enrichment	59	3.2	9.1
50	Nitrogen-Air Mixture of 19 percent Oxygen	44	4.0	5.4

Fermentation Conditions: 400 rpm, 30°C, pH 7.0

[1]Oxygen Enrichment to Maintain 0.05 atm. Dissolved Oxygen

d. Behavior of the Oxygen Uptake Rate During the Period of Oxygen-Enriched Aeration

Data summarized in figure 19 shows the OUR, oxygen tension, and inlet and effluent oxygen concentrations during the period of oxygen enrichment used in the fermentation shown in figure 18. The observed OUR increased abruptly for the first two hours reaching a value of almost 2.5 times the value observed at the onset of oxygen enrichment. The OUR then decreased rapidly over the next four hours as oxygen enrichment of the aeration continued.

C. Production of Neomycins by Streptomyces fradiae 3535

1. Neomycin: Chemistry and Commercial Production

Neomycin, a group of aminoglycoside antibiotics produced by Streptomyces fradiae, is composed of approximately six components of varying antimicrobial activity. (figure 20) The three main components are neomycins A, B, and C. Neomycin B is the major component with the greatest antibiotic activity; neomycin C is an isomer of B; and neomycin A, also called neamine, is a moiety of both B and C. Mono-acetly derivatives of neomycin B and C (LP_B, LP_C) are present in the fermentation but show no antimicrobial activity (62). Neomycins D, E, and F may also be present in commercially produced neomycin. these appear in varying amounts in the fermentation and usually represent less than one percent of the total product (86). These three compounds have been shown to be identical with paromamine, paromomycin I and paromomycin II respectively (87). The appearance of neomycin B-glycoside in the fermentation when S. fradiae is grown in the presence of three to nine percent glucose has also been reported (88).

Neomycin appears to be an integral component of the mycelium of

FIGURE 19

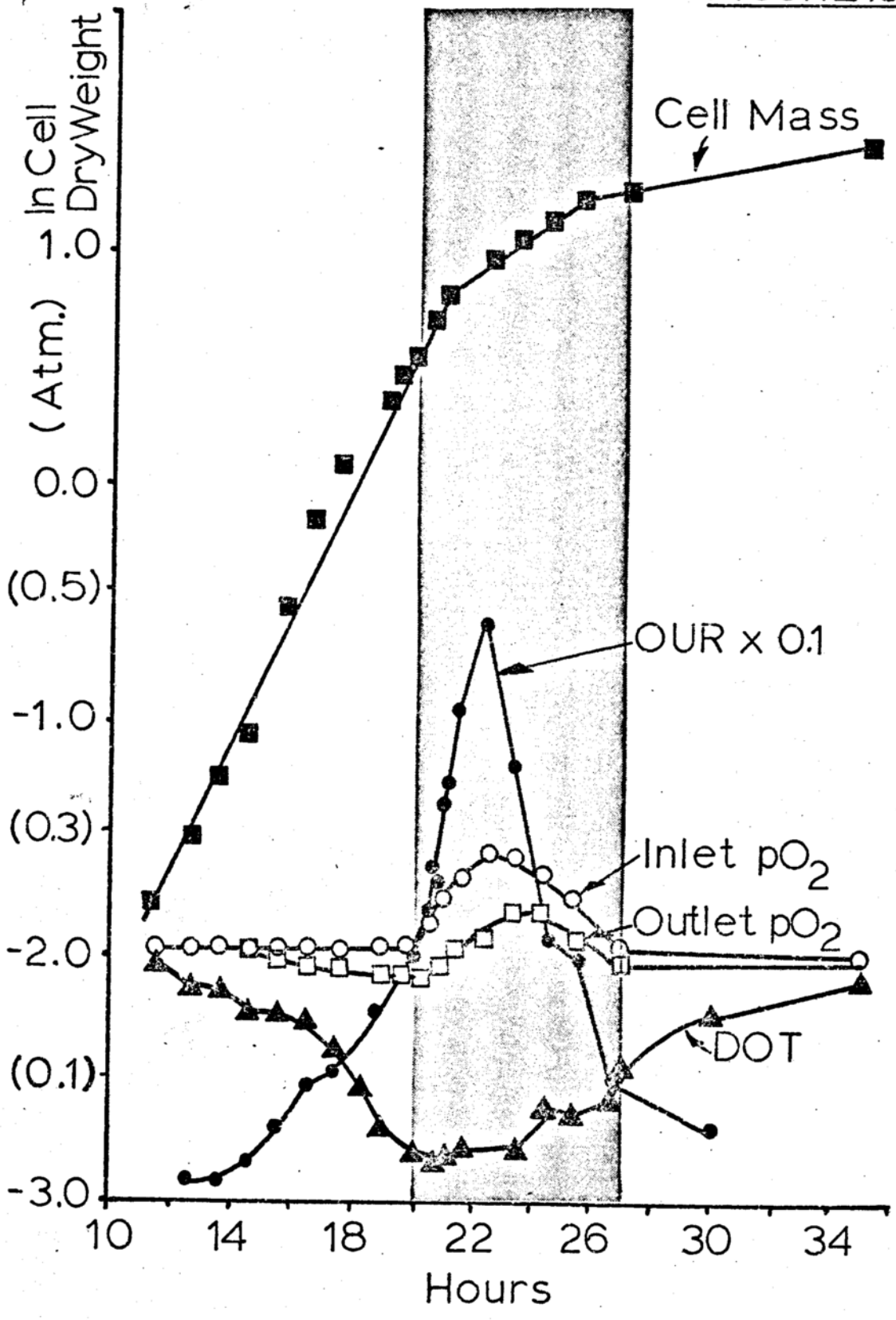
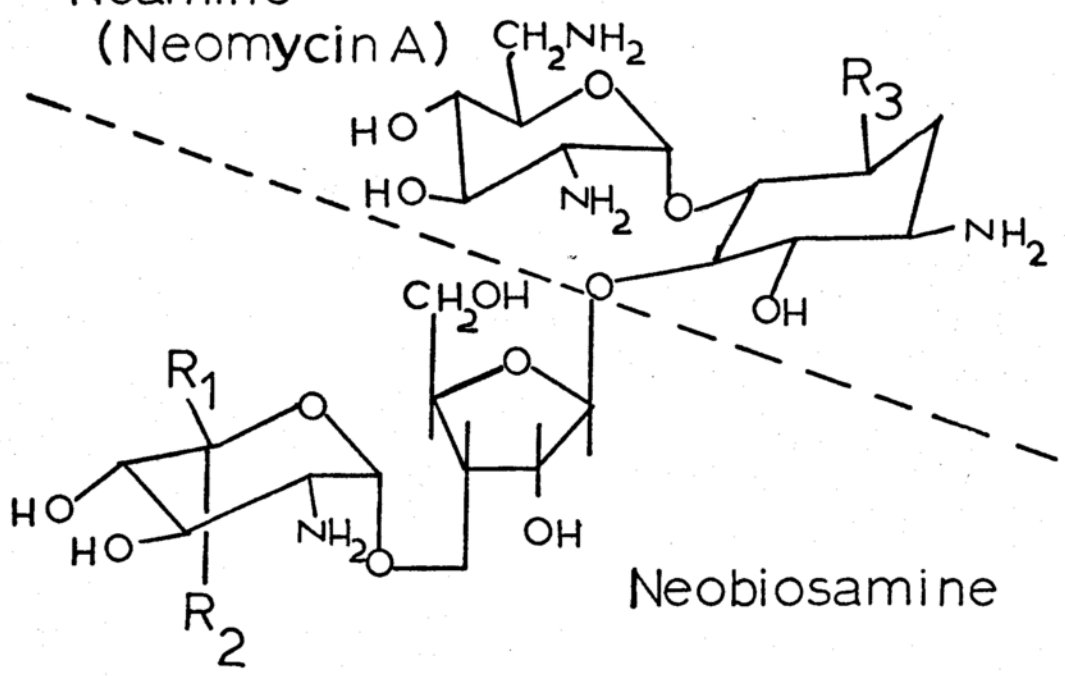
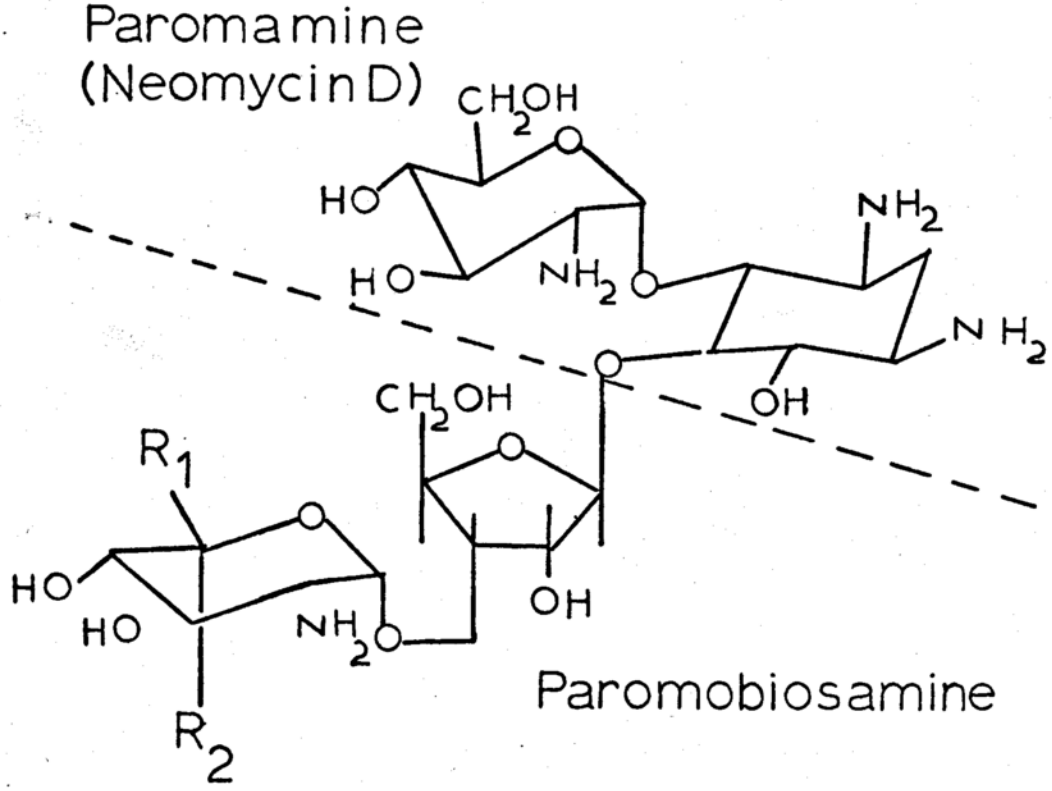

ln Cell Dry Weight
1.0
0.0
(0.5)
-1.0
-2.0
-3.0
(Atm.)
(0.3)
(0.1)
Cell Mass
OUR x 0.1
Inlet pO2
Outlet pO2
DOT
10
14
18
22
26
30
34
Hours

FIGURE 20


Neamine
(Neomycin A)
Neobiosamine


Paromamine
(Neomycin D)
Paromobiosamine

S. fradiae and is thought to be released from the cells during autolysis (86).

Methods for the production of high levels of neomycin are well guarded industrial secrets. Several studies have appeared in the literature on the utilization of various medium components during the growth of S. fradiae. Both defined and complex media have been studied for neomycin production. (89,90,91,92) Industrial media for the production of this antibiotic are composed of various dextrin, starch, soy protein, or powdered yeast preparations (86,91). Glucose has been found to inhibit the production of high titers (93) while maltose and dextrins are good carbon sources (89). Medium pH, incubation temperature, and aeration have all been shown to affect neomycin production (89,91) and have been manipulated in order to try to direct the fermentation toward production of neomycin B with only a low level of neomycin C formation.

2. Choice of Production Medium

Complex nutrients for the neomycin production medium were tested using 200 ml. of medium in 2000 ml. Erlenmeyer flasks at 30°C on a 300 rpm rotary shaker. Neomycin titers were followed by bioassay during 100 hours of incubation. Soybean meal (Archer Daniel Midlands), toasted and non-toasted soy flour (ADM 63-145, 63-100), and toasted and non-toasted soy grits (ADM 63-380,63-770) were examined at a concentration of 30 gms./liter in a medium containing 30 gms./liter dextrin (Stadex 60K A. E. Staley). Soybean meal supported the best production of approximately 0.6 grams per liter under these conditions. Glucose could be used in the inoculum medium (10 gms./liter) if calcium carbonate was included so that the pH would not drop below pH 6. No calcium carbonate was required in the production medium as dextrin

is utilized by S. fradiae more slowly than glucose resulting in a more gradual decrease in pH during the first 40 hours of incubation.

3. Effect of pH Control on Production

It would be easier to interpret the effects of oxygen-enriched aeration on the physiology of S. fradiae, CO_2 evolution, and neomycin production if the pH of the medium could be maintained at a constant level during the course of the fermentation. Figure 21 shows the results of two 25 liter neomycin fermentations at high impeller speed, 400 rpm, and high aeration rate, 1.0 vvm. The pH of one of the fermentations was controlled at pH 7.0 using aseptic addition of 2 N H_2SO_4.

In the fermentation without pH control the pH decreases from pH 6.8 to pH 6.0 during the first 45 hours of the incubation followed by a gradual rise to a final value of pH 8.5 after 100 hours. The data in figure 21 indicates that antibiotic was produced during the first 45 hours of incubation regardless of whether the pH was allowed to drop or was controlled at pH 7.0. However when the steady increase in pH during the last 50 hours of the fermentation was eliminated, antibiotic production stopped. Utilization of dextrin from the medium also appeared to stop at the point (50 hours) where antibiotic production ceased

Since no information has appeared in the literature on the optimum level of pH control for the production of neomycin by S. fradiae nor is there any data to suggest that pH control is being used in the industrial production of this antibiotic, the effect of oxygen-enriched aeration was investigated without conditions of controlled pH.

4. Effect of Aeration Rate on Production at Constant Impeller Speed

The production of neomycin could be increased by increasing the aeration rate without increasing the impeller speed (figure 22).

FIGURE 21

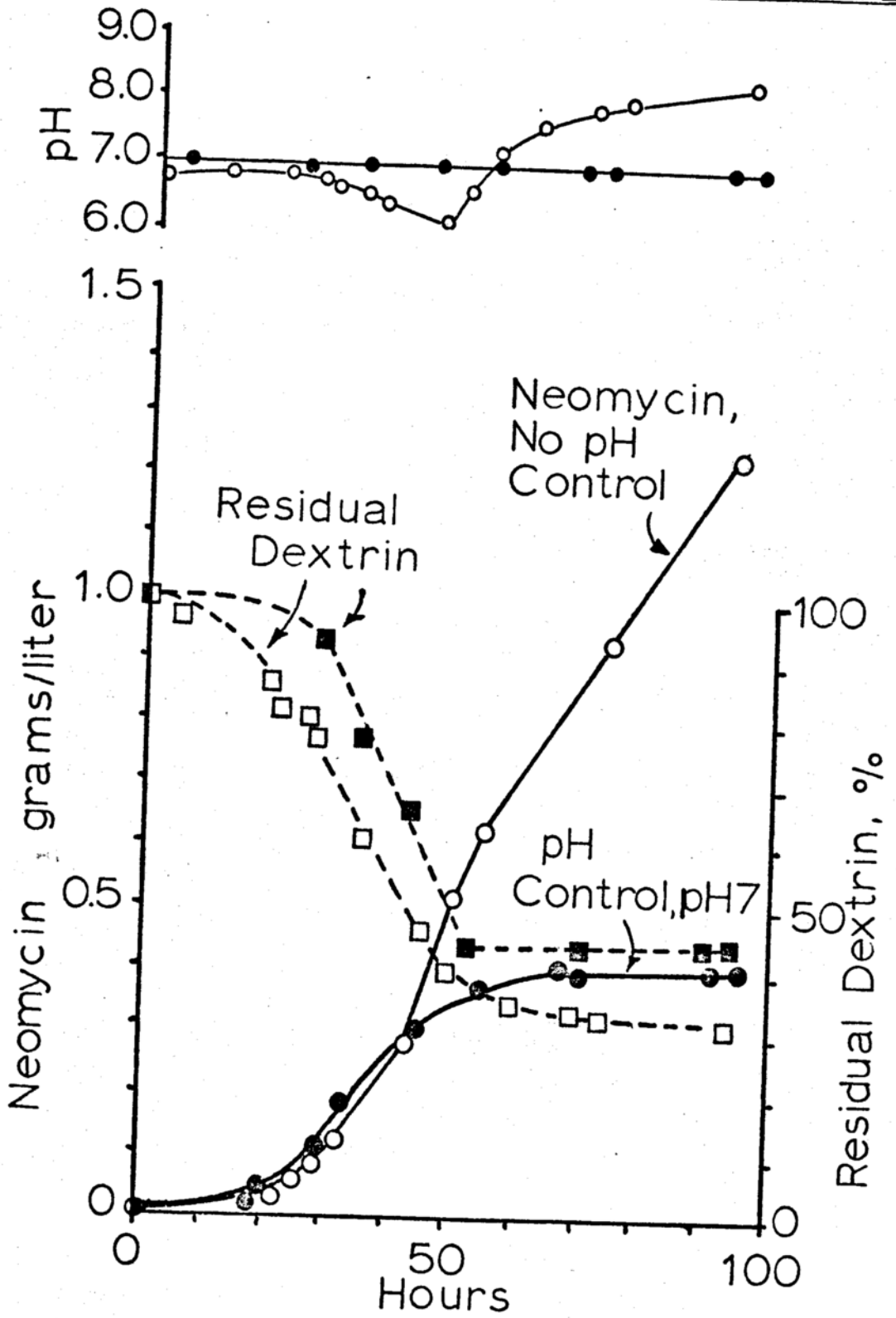
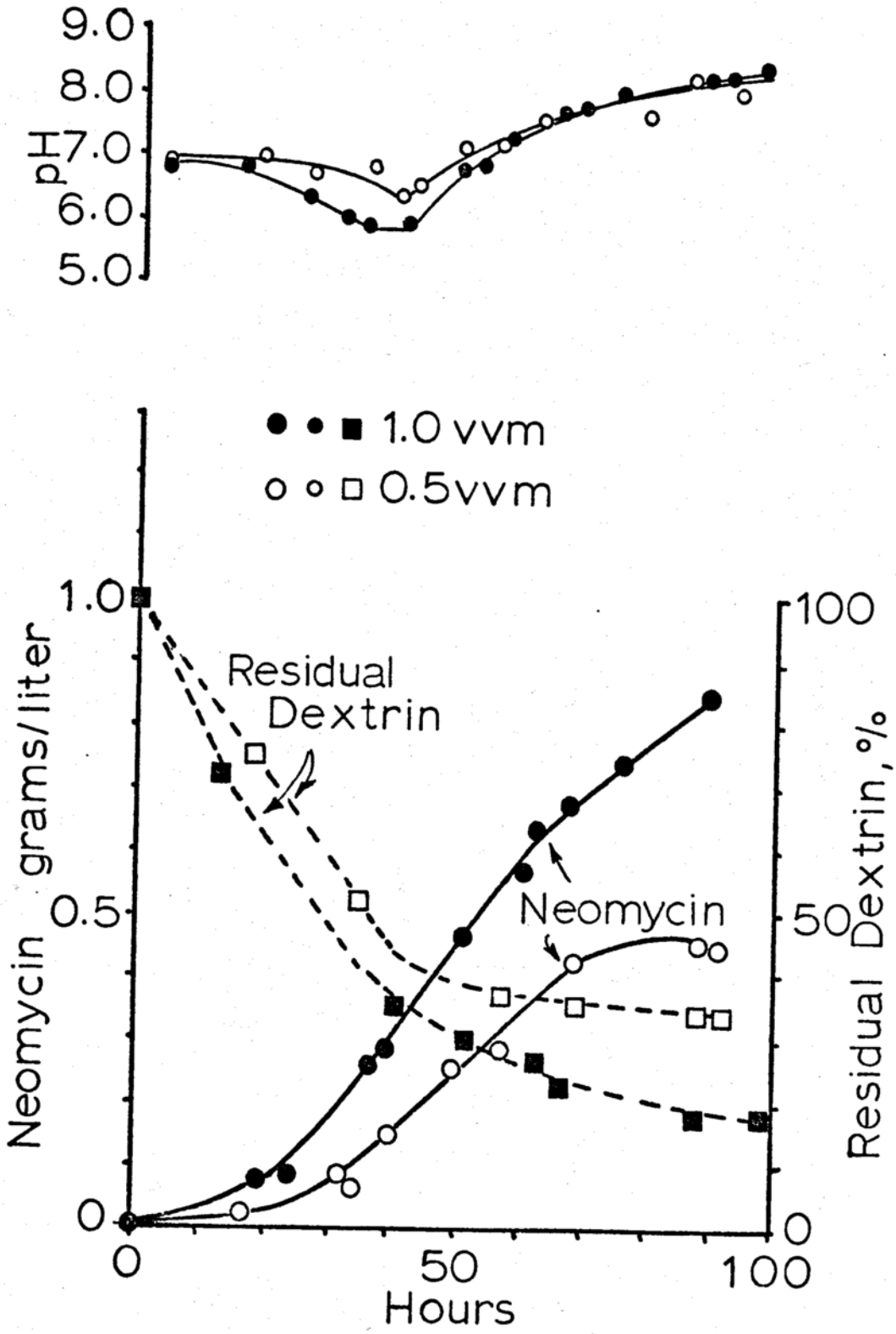

9.0
8.0
pH 7.0
6.0
1.5
1.0
0.5
0
Neomycin, grams/liter
Residual Dextrin
Neomycin, No pH Control
pH Control, pH7
100
50
0
Residual Dextrin, %
0
50
100
Hours

FIGURE 22


9.0
8.0
pH 7.0
6.0
5.0
● ● ■ 1.0 vvm
○ ○ □ 0.5vvm
1.0
0.5
0
Neomycin grams/liter
Residual Dextrin
Neomycin
100
50
0
Residual Dextrin, %
0
50
100
Hours

Increasing the aeration rate from 0.5 volumes of air per volume of medium per minute (vvm) to 1.0 vvm resulted in a two fold increase in the yield of antibiotic after 100 hours of incubation. Sufficient oxygen was transferred at both 1.0 and 0.5 vvm with 400 rpm impeller speed so that neither fermentation became oxygen limited during 100 hours of incubation. Aeration at 0.5 vvm was chosen as the level for investigation of the effect of oxygen-enriched aeration. This low aeration rate more closely represents the level of aeration employed in large scale production utilizing a medium containing three percent dextrin (86).

5. The Effect of Oxygen-Enriched Aeration on Production of Neomycin

The effect of oxygen-enrichment on the production of neomycin was studied at four different levels of power input. Impeller speeds of 400 rpm, 300 rpm, 200 rpm, and 100 rpm were used with an aeration rate of 0.5 vvm. These impeller speeds represent relative oxygen transfer rates of 1.0, 0.6, 0.2 and 0.05 respectively as determined by the Glucose Oxidase method (section A).

Oxygen-enrichment of the aeration was made in response to physiological oxygen demand. Compressed oxygen was added to the aeration line beginning at the point in the fermentation where the dissolved oxygen tension (DOT) reached a level of 0.05 atmospheres. Only enough oxygen was added to maintain this 0.05 atm. level. As the oxygen demand of the culture decreased, the amount of oxygen-enrichment was correspondingly decreased so that the 0.05 atm. level would not be exceeded. Both manual and automatic methods of oxygen-enrichment were used. (Methods, section C)

In the following figures the shaded area denotes the period of oxygen-enrichment - constant DOT at 0.05 atmospheres.

a. Respiration and pH Behavior of S. fradiae 3535

Figures 23, 24, 25, and 26 show the effect of oxygen-enriched aeration on the evolution of gas-phase CO_2 during the production of neomycin by S. fradiae 3535. This data is summarized in table X. At lower impeller speeds (100-200 rpm) significantly more CO_2 was produced when oxygen-enrichment was used. At these lower impeller speeds oxygen-enrichment was required during the majority of the fermentation period. The increased pO_2 of the sparge gas resulted in better culture respiration as evidenced by increased CO_2 evolution. The pH behavior of the oxygen-enriched fermentations at 100 rpm and 200 rpm was significantly different than when sparging with air alone (figure 27). At low impeller speeds oxygen transfer was poor. As expected, O_2 enrichment increased the driving force of oxygen transfer resulting apparently in better culture viability.

At 300 rpm and 400 rpm impeller speeds the application of air + oxygen did not significantly increase the CO_2 evolution nor did it appear to change the pH behavior of the fermentation.

The average respiratory quotient (R.Q.) during the period of oxygen-enrichment increased under conditions of better oxygen transfer from 0.6 ± 0.05 at 300 rpm to 1.0 ± 0.05 at 400 rpm. Manual control of O_2 enrichment which allowed for ± 0.015 atm. fluctuations in the dissolved oxygen tension did not result in a significant increase in the R.Q. during the period of enrichment. The average value at 300 rpm impeller speed was 0.7 ± 0.1 for both air and air + O_2. At 400 rpm impeller speed the average R.Q. was approximately 1.0 ± 0.05 during the period of oxygen enrichment

FIGURE 23

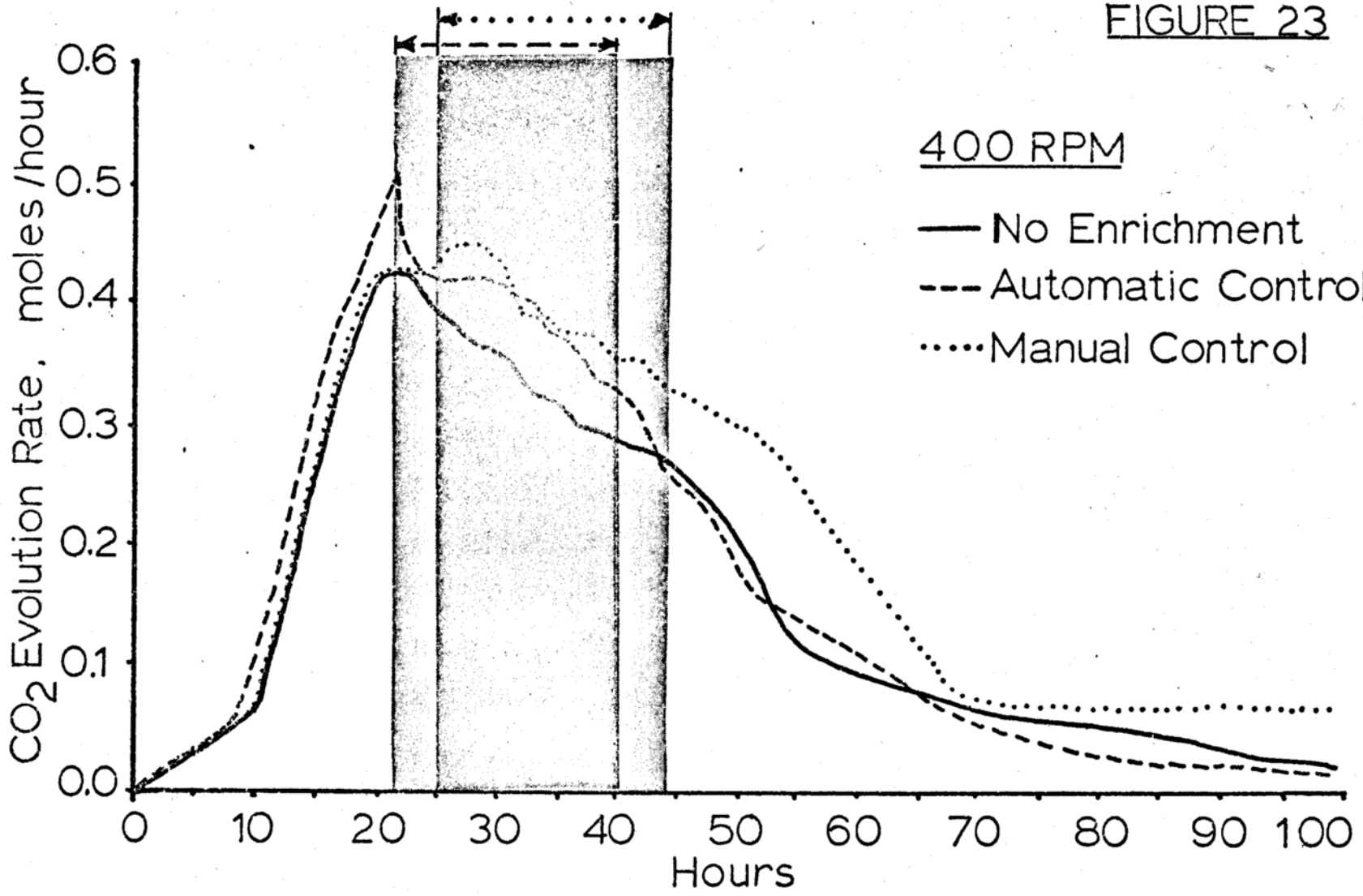

400 RPM
No Enrichment
Automatic Control
Manual Control
CO_2 Evolution Rate, moles/hour
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40
50
60
70
80
90
100
Hours

FIGURE 24

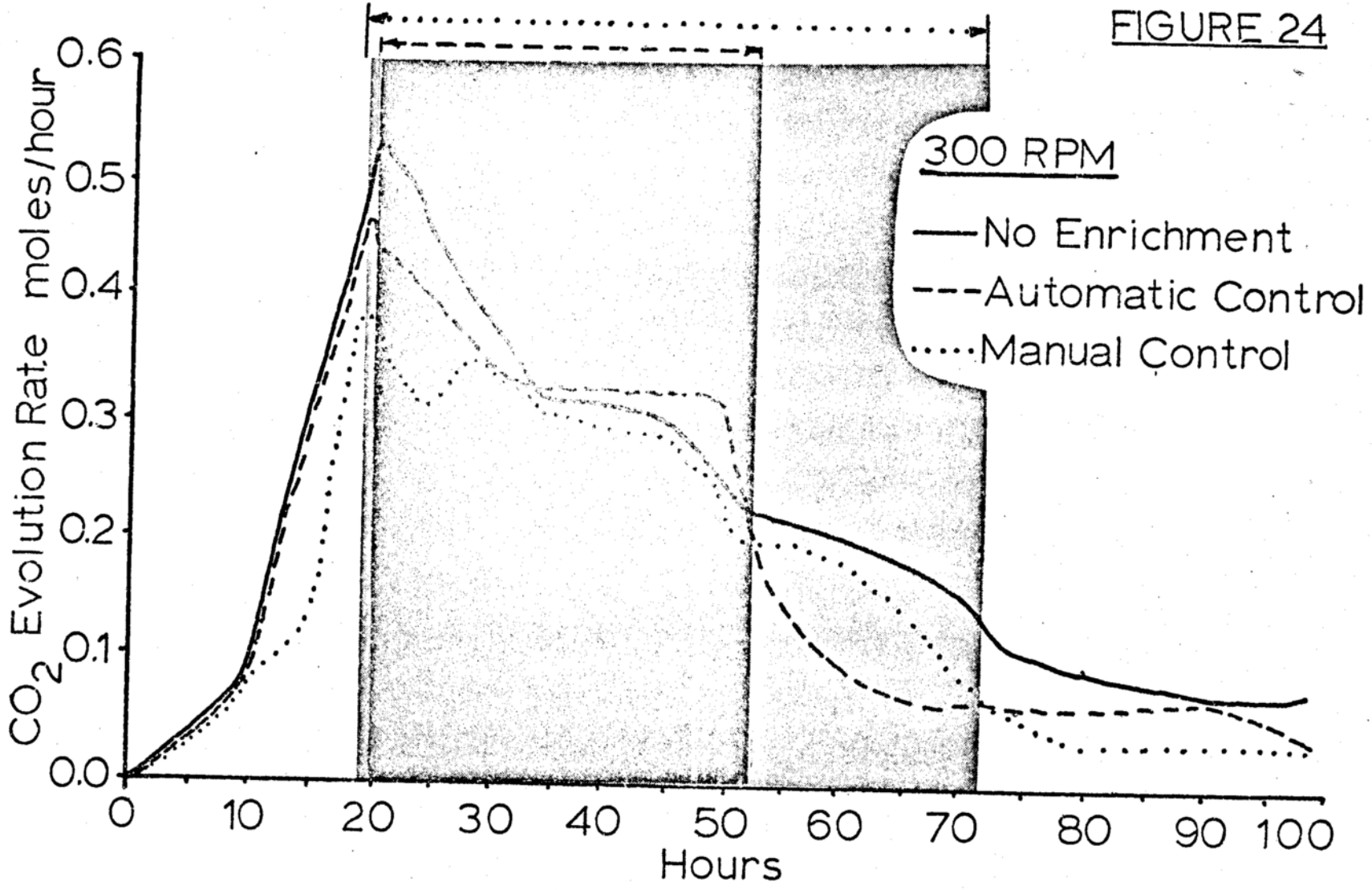

300 RPM
No Enrichment
Automatic Control
Manual Control
CO_2 Evolution Rate moles/hour
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40
50
60
70
80
90
100
Hours

FIGURE 25

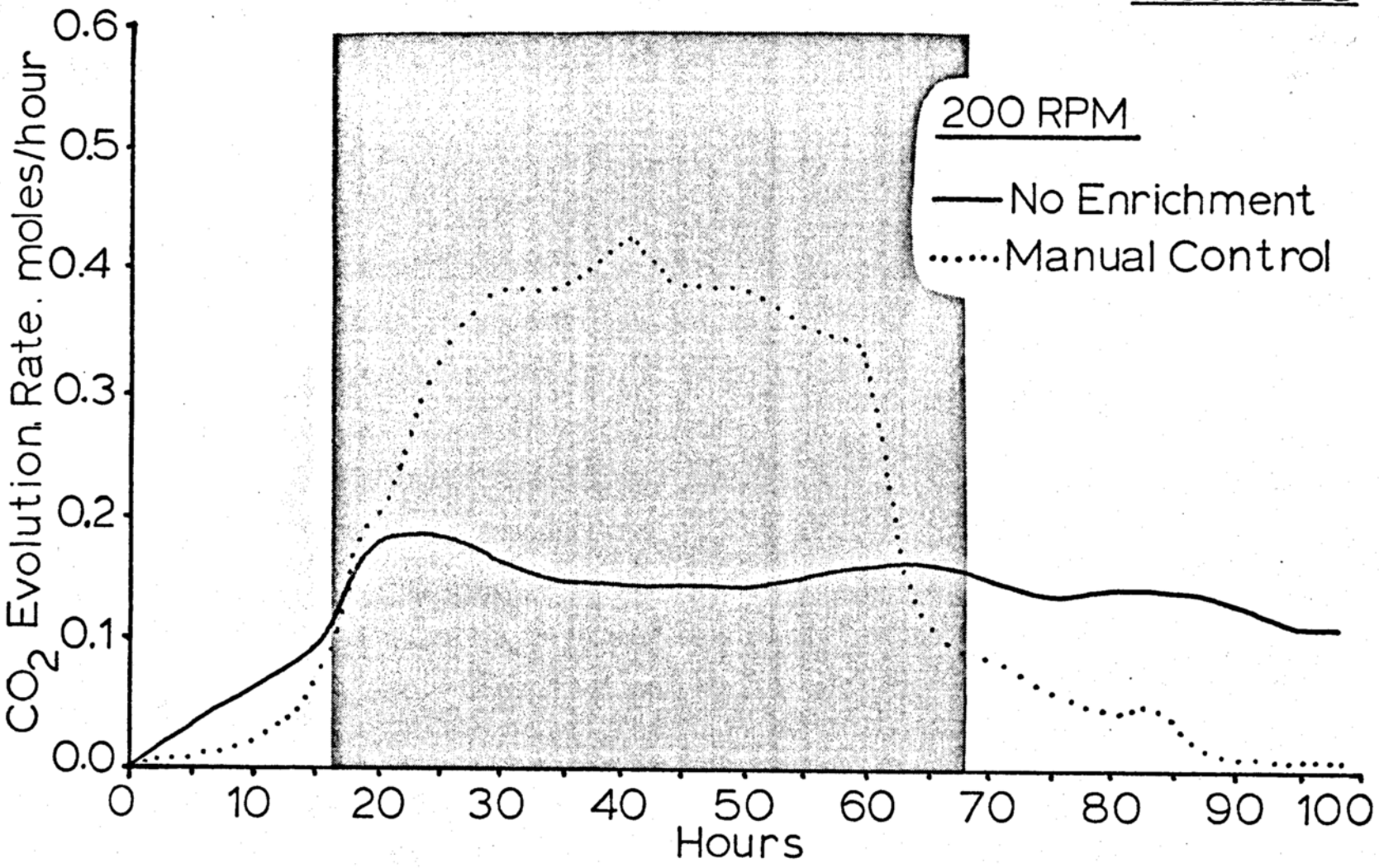

200 RPM
No Enrichment
Manual Control
CO_2 Evolution Rate, moles/hour
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40
50
60
70
80
90
100
Hours

FIGURE 26

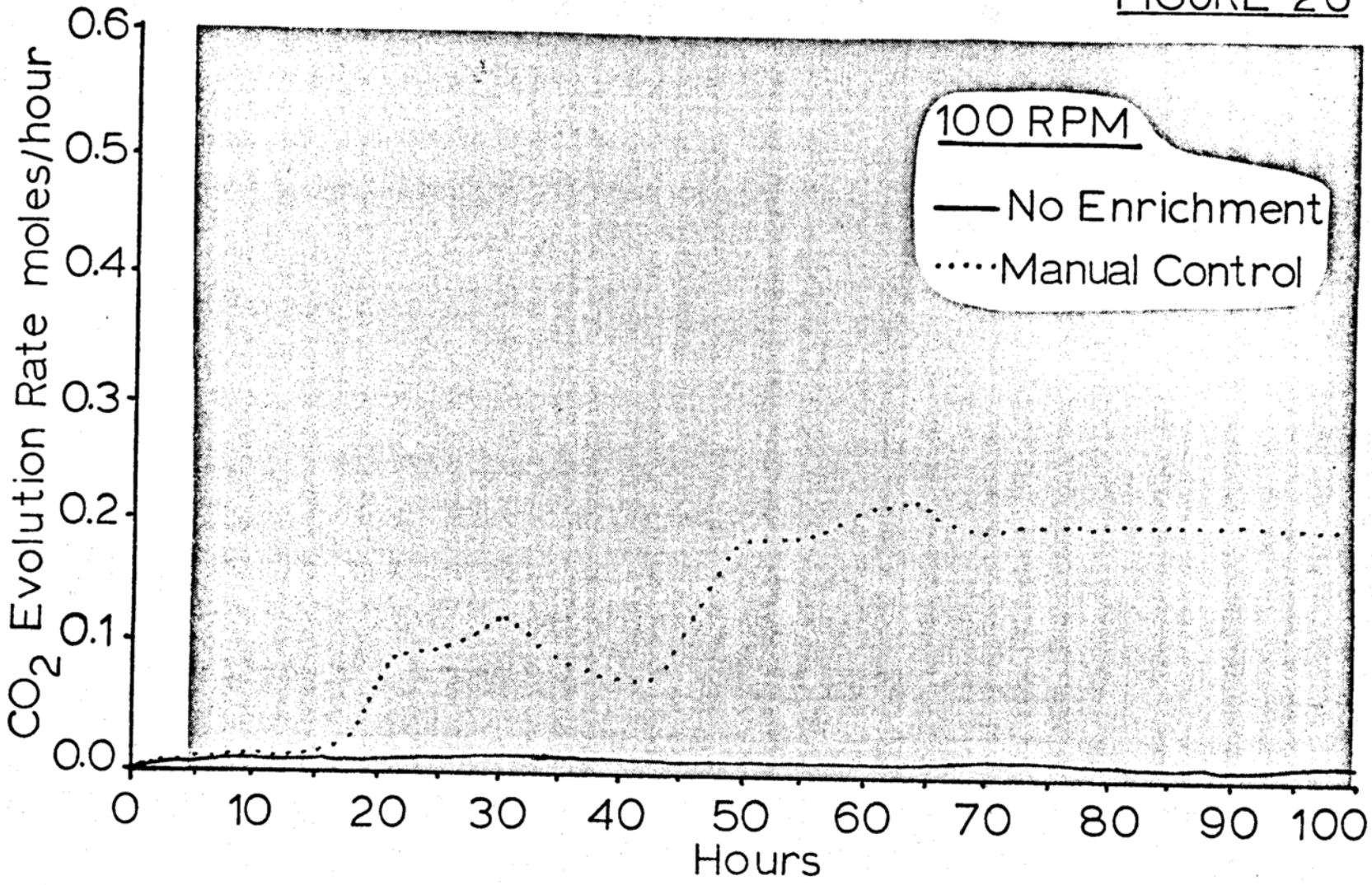
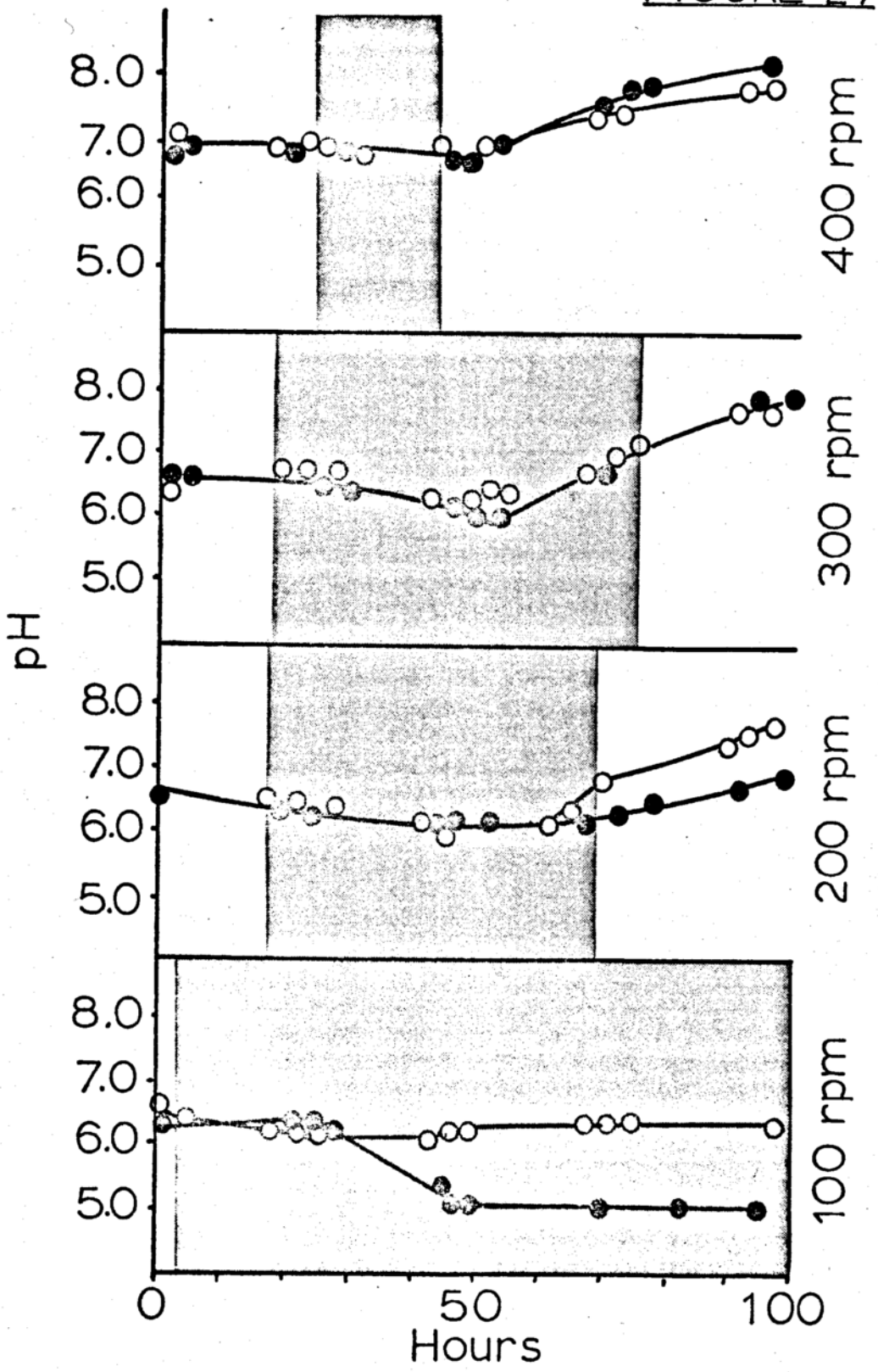

100 RPM
No Enrichment
Manual Control
CO_2 Evolution Rate moles/hour
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40
50
60
70
80
90
100
Hours

Table X SUMMARY OF CO_2 EVOLUTION BY S. fradiae FERMENTATIONS

IMPELLER PPM	ENRICHMENT (0.05 atm. DOT)	TOTAL GAS-PHASE CO_2	CO_2 DURING ENRICHMENT	CO_2 DURING EQUIVALENT PERIOD	RELATIVE CO_2	AVE. (P_g-P_l) DURING ENRICHMENT	AVE. R.Q. DURING EQUIVALENT PERIOD	AVE. R.Q. DURING ENRICHMENT
100	None	1.14	-	-	1.0	0.21	Not Determined	-
100	Manual	14.7	14.7	1.14	12.9	0.21	Not Determined	-
200	None	15.0	-	-	1.0	0.19	Not Determined	-
200	Manual	19.7	17.9	9.22	1.94	0.35	Not Determined	-
300	None	21.7	-	-	1.0	0.15	-	-
300	Manual	17.4	14.0	16.2	0.86	0.22	0.73	0.77
300	Automatic	19.9	12.3	11.9	1.0	0.23	0.58	0.41
400	None	18.1	-	-	1.0	0.08	-	-
400	Manual	28.8	8.4	6.9	1.22	0.11	1.1	1.1
400	Automatic	19.0	8.2	7.3	1.12	0.18	1.2	1.7

FIGURE 27



for both air and O_2 enriched fermentations. Automatic addition ($\pm$ 0.005 atm. fluxuations in DOT) decreased the averate R.Q. to 0.4 $\pm$ .1 at 300 rpm but resulted in an increased R.Q. at 400 rpm of approximately 1.5 $\pm$ 0.2.

Compressed oxygen did not visibly appear to effect mycelial integrity during these fermentations. Fragmentation of the mycelium occurred only under very poor oxygen transfer conditions (100 rpm - air sparge). This fragmentation resulted in the abnormal drop in pH to pH 5.1 during this oxygen limited fermentation. Oxygen enrichment under these conditions maintained the mycelium intact resulting in a higher final pH of 6.6. Sporulation of the Streptomycete was not monitored.

b. Increasing Antibiotic Yield at Constant Impeller Speed

Figure 28 and table XI summarize the effect of oxygen-enriched aeration at various impeller speeds on the final titer of neomycins. Both 100 hour and 164 hour fermentation periods were investigated. The amount of additional antibiotic obtained with the 64 hour longer fermentation was only 0.1 to 0.2 grams per liter either with or without O_2 enrichment.

In all cases the yield of bioactive neomycins per gram of dextrin utilized was increased by application of oxygen-enrichment. However, the increased yield of neomycins at low impeller speeds (100-200 rpm) is probably due to the effect of increased pO_2 on culture viability as evidenced by a more 'normal' pH behavior. At 300 rpm and 400 rpm impeller speeds the pH behavior of the air sparged and air + O_2 sparged fermentations was identical. The increased antibiotic yield under these conditions can be attributed to the application of oxygen-enrichment.

FIGURE 28

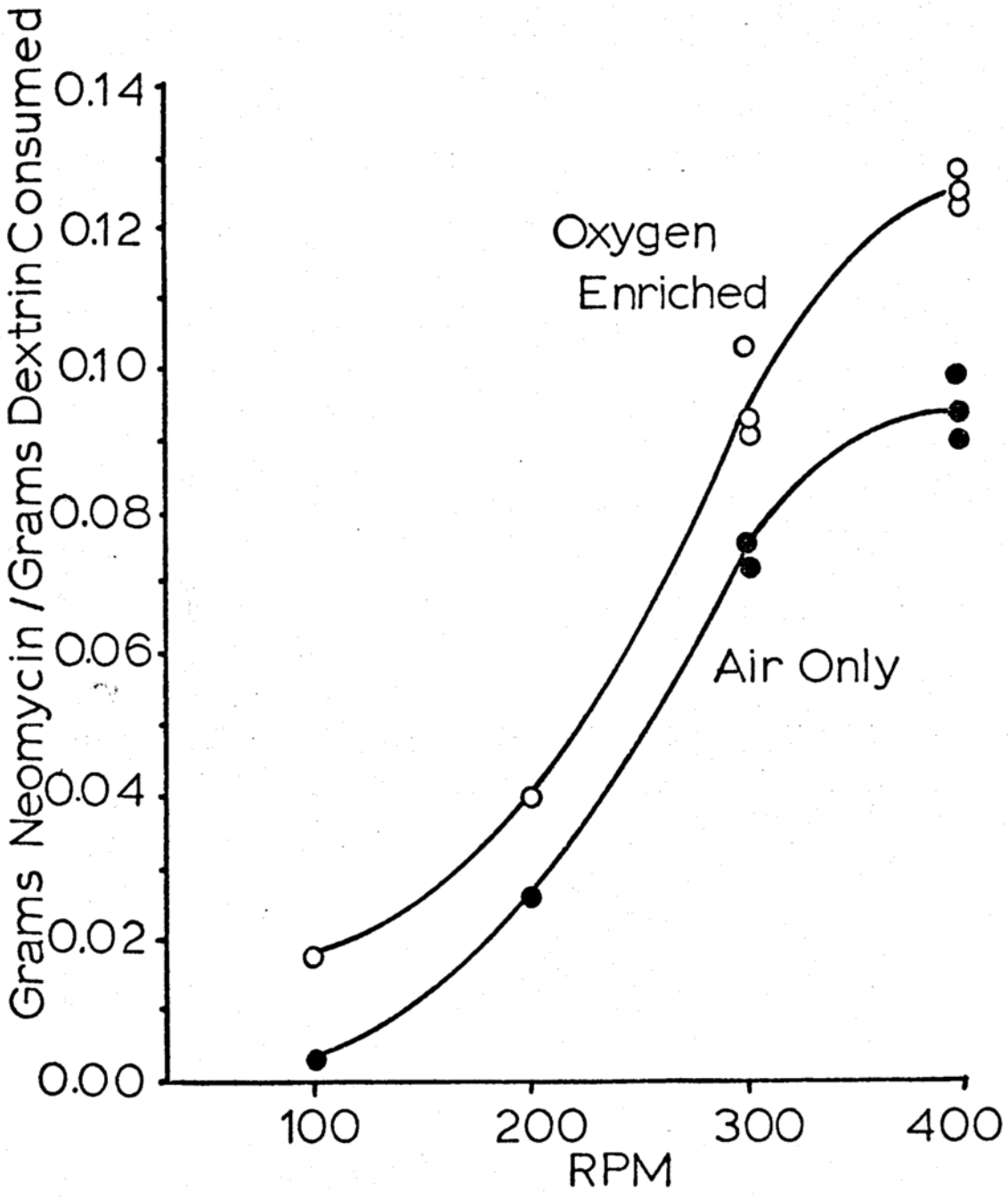

Grams Neomycin / Grams Dextrin Consumed
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Oxygen
Enriched
Air Only
100
200
300
400
RPM

Table XI

SUMMARY OF THE INCREASED YIELD OF NEOMYCINS IN RESPONSE TO OXYGEN ENRICHED AERATION AT VARIOUS IMPELLER SPEEDS

- 0.5 vvm aeration rate -

IMPELLER RPM	METHOD OF O_2 ENRICHMENT	ENRICHMENT TIME IN HOURS	psig O_2 DEMANDED TO MAINTAIN 0.05 ATM. D.O.	LENGTH OF INCUBATION IN HOURS	YIELD OF NEOMYCIN IN GMS. PER LITER AS SULFATE +
100	None	0	0	100	0.041
100	None	0	0	100	0.030
100	*Manual	88	10,500	100	0.186
200	None	0	0	100	0.495
200	Manual	51	2,700	100	0.891
300	None	0	0	100	1.48
300	Manual	55	1,900	100	2.33
300	**Automatic	34	950	164	1.91
400	None	0	0	100	1.68
400	Manual	19	100	100	2.40
400	None	0	0	164	1.83
400	Automatic	19	200	164	2.43

*MANUAL OXYGEN ENRICHMENT: Continuous enrichment at constant total aeration rate of 0.5 vvm to maintain 0.05 atm. dissolved oxygen tension. O_2 flow rate varied in response to demand.

**AUTOMATIC OXYGEN ENRICHMENT: O_2 added in shots at 5 liters/minute in response to oxygen demand to maintain 0.05 atm. D.O.T.

+ NEOMYCIN SULFATE POTENCY: 655 mg base/gm.

Table XI also shows that the length of time required for oxygen-enrichment decreases from 88 percent to less than 20 percent of the total fermentation period as the impeller speed (and resulting oxygen transfer rate) is increased from 100 rpm to 400 rpm. During 164 hours of incubation, the period of oxygen-enrichment required for 300 rpm was 34 hours and only 19 hours at 400 rpm. As a result of these relatively short period of O_2 enrichment, a 16 to 25 percent increase in the final titer was realized.

The yield of neomycin on oxygen ($Y_{neomycin_{O_2}}$) defined as the grams of antibiotic produced per gram of oxygen taken up by the culture was found to be between 0.03 and 0.044 at 300 rpm impeller speed and 0.063 to 0.072 at 400 rpm. The method of oxygen-enrichment did not significantly affect $Y_{neomycin_{O_2}}$ values.

c. The Rate of Neomycin Biosynthesis

Figures 29 and 30 compare the production of neomycin during 164 hours of incubation with and without O_2 enrichment of the aeration. The 0.3 and 0.6 grams per liter increase in antibiotic is the result of the rate of antibiotic production being affected by O_2 enriched aeration. With air + O_2 the level of antibiotic produced at 40 hours is about the same as the level after 70 hours of air sparging under the same conditions of aeration rate and impeller speed. The maximum yield of antibiotic obtained with air sparging is reached at 117 hours of incubation. The same level of neomycin can be obtained, with oxygen-enrichment, after 60 hours of incubation. This would imply that the overall fermentation time could be cut in half.

Table XII summarizes the effect of O_2 enrichment on the rate of neomycin production during the period of oxygen-enrichment for the data from figures 29 and 30. O_2 enrichment resulted in an increased

FIGURE 29

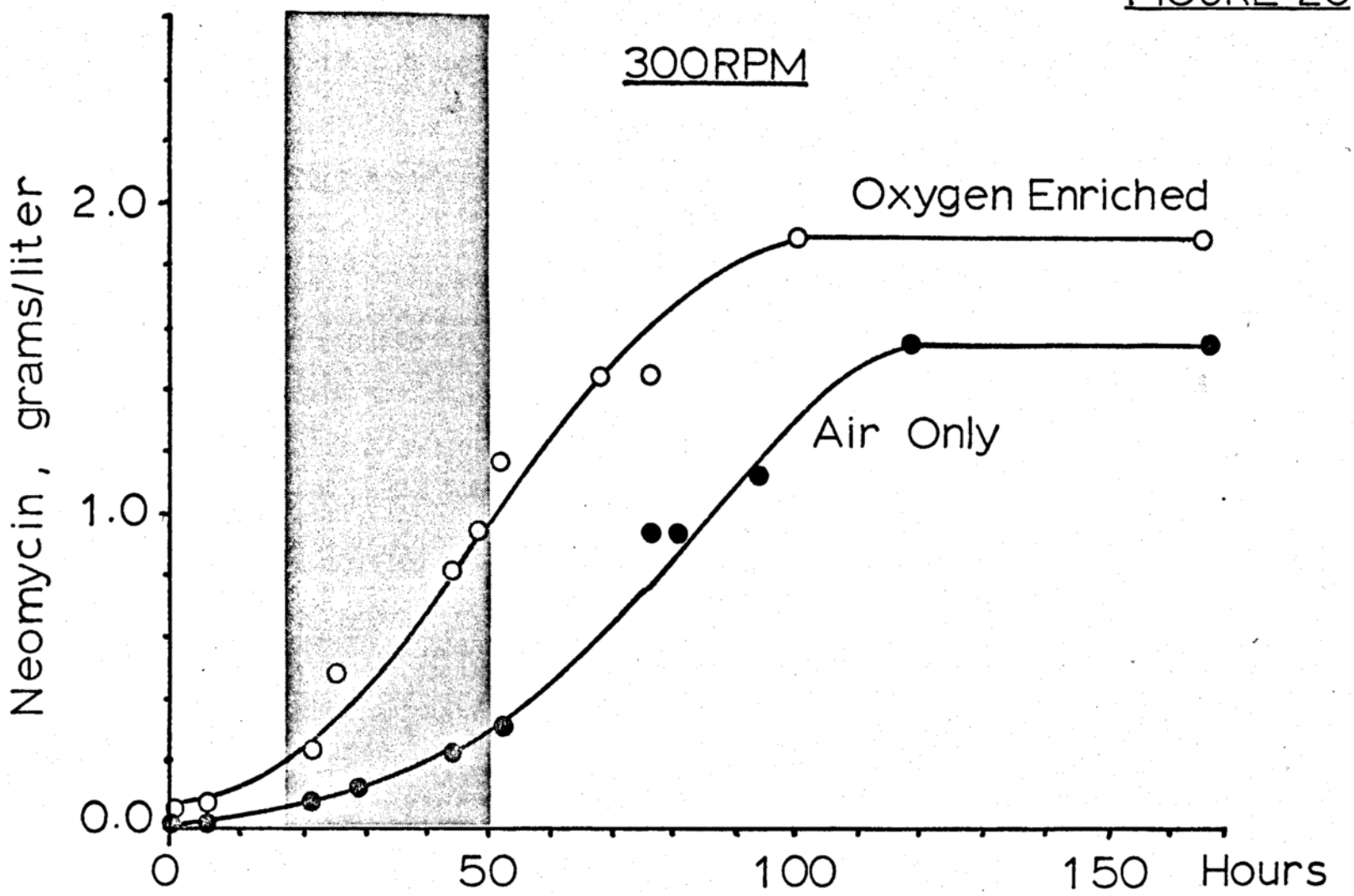

300RPM
Oxygen Enriched
Air Only
Neomycin, grams/liter
2.0
1.0
0.0
0
50
100
150
Hours

FIGURE 30

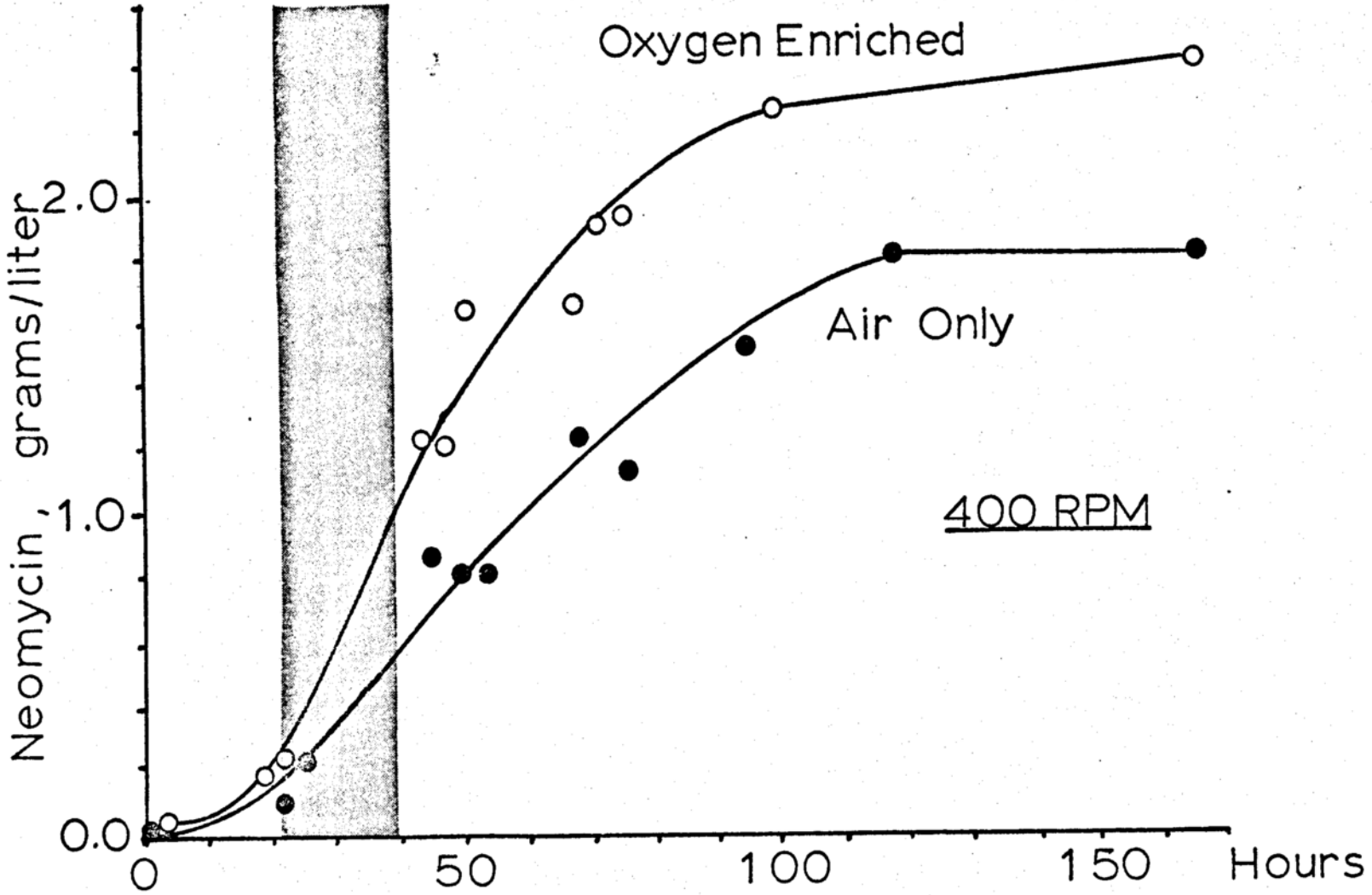

Oxygen Enriched
Air Only
400 RPM
Neomycin, grams/liter
2.0
1.0
0.0
0
50
100
150
Hours

Table XII

THE EFFECT OF THE METHOD OF OXYGEN ENRICHMENT ON THE RATE OF NEOMYCIN BIOSYNTHESIS DURING THE PERIOD OF OXYGEN ENRICHMENT

-0.5 vvm initial aeration rate-					
Impeller rpm	Method of O_2 Enrichment	Final Yield of Neomycin in gms./25 l	Moles of Enriched O_2 Used	Percent added O_2 Used	Grams Neomycin per Hour in 25 l. during period of Enrichment
300	None	37.0	0	0	0.21
300	Manual	58.3	222	6	0.96
300	Automatic	47.8	111	8	0.72
400	None	42.0	0	0	0.63
400	Manual	60.0	11.6	23	1.4
400	Automatic	60.8	23.3	3	1.1

production rate of 2 to 4.5 fold over the air sparged fermentations during the corresponding period of time.

The time course data of figures 29 and 30 also suggest that when O_2 enrichment is applied the maximum level of antibiotic is obtained between 90 and 100 hours of incubation. Antibiotic accumulation ceases at this point probably due to some nutrient limitation other than oxygen. Continuation of neomycin accumulation after 100 hours could be obtained by better medium design.

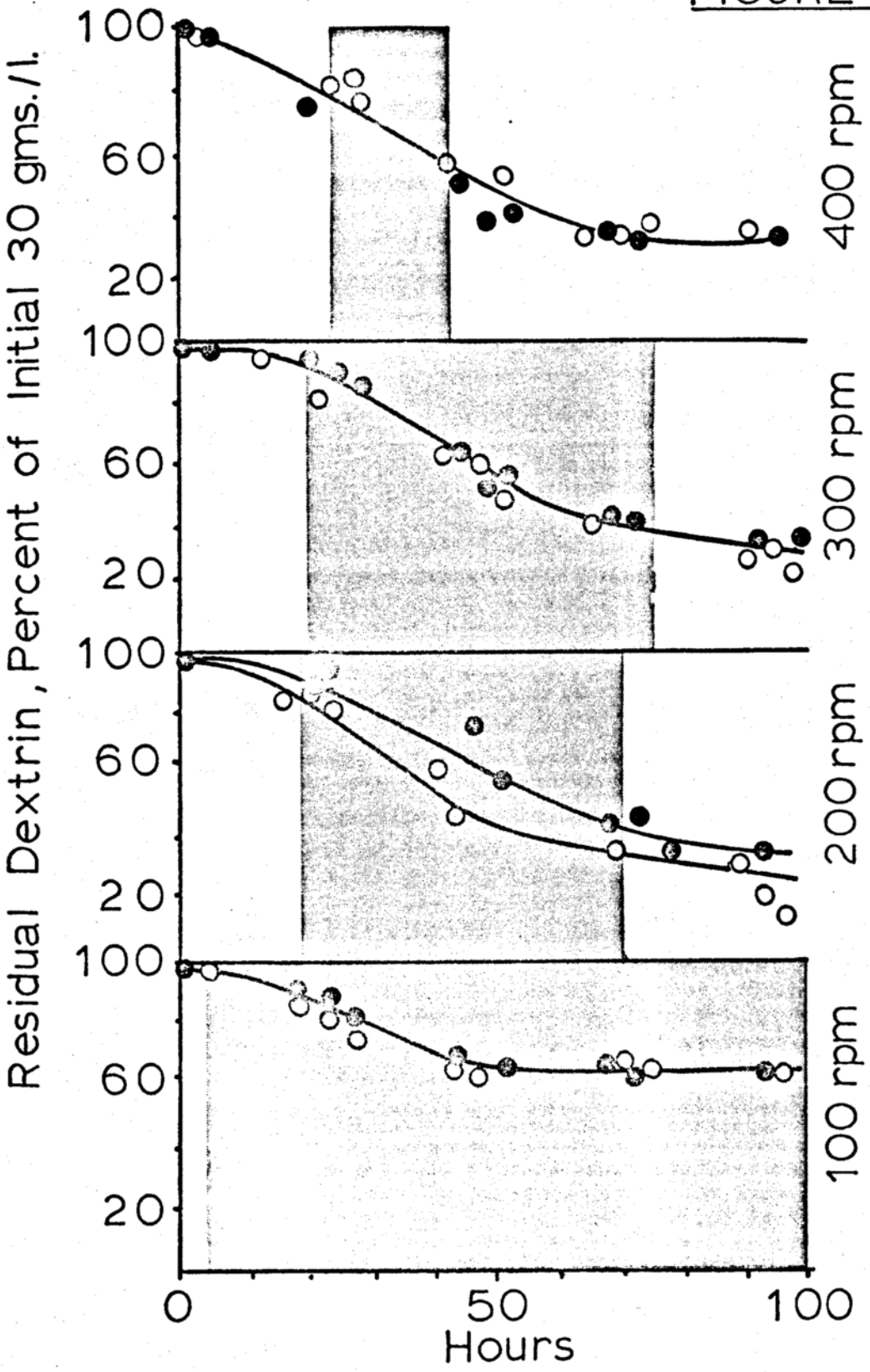
d. Utilization of Medium Carbohydrate

The majority of industrial antibiotic fermentations utilize inexpensive complex nutrients in large quantities. The percentage of conversion of carbon source into cell mass or antibiotic is probably low resulting in a significant portion of the nutrients remaining in the medium at the end of the fermentation. Increased utilization of added nutrients would mean a significant savings of medium costs per kilogram of antibiotic produced.

Data was collected from the S. fradiae fermentations in order to determine whether O_2 enrichment could favorably affect more complete utilization of medium dextrin. Figure 31 summarizes the time course data of dextrin utilization at various impeller speeds. Oxygen-enrichment did not significantly decrease the amount of dextrin remaining at the end of the fermentation from that using air sparging alone. This residual dextrin may not be utilizable by this organism. O_2 enrichment also did not affect the rate of dextrin utilization by this organism.

Dextrin is utilized by S. fradiae presumably by the production of an extracellular hydrolytic enzyme cleaving the polysaccharide into monosaccharide units. Because of this method of carbon source assimilation, O_2 enrichment may have to affect the activity of the hydrolytic

FIGURE 31


Residual Dextrin, Percent of Initial 30 gms./l.
100
60
20
400 rpm
300 rpm
200 rpm
100 rpm
0
50
100
Hours

enzymes or their synthesis in order to affect dextrin utilization. A more rapid utilization of the available carbon source may be realized with oxygen-enrichment when S. fradiae is grown on glucose instead of dextrin.

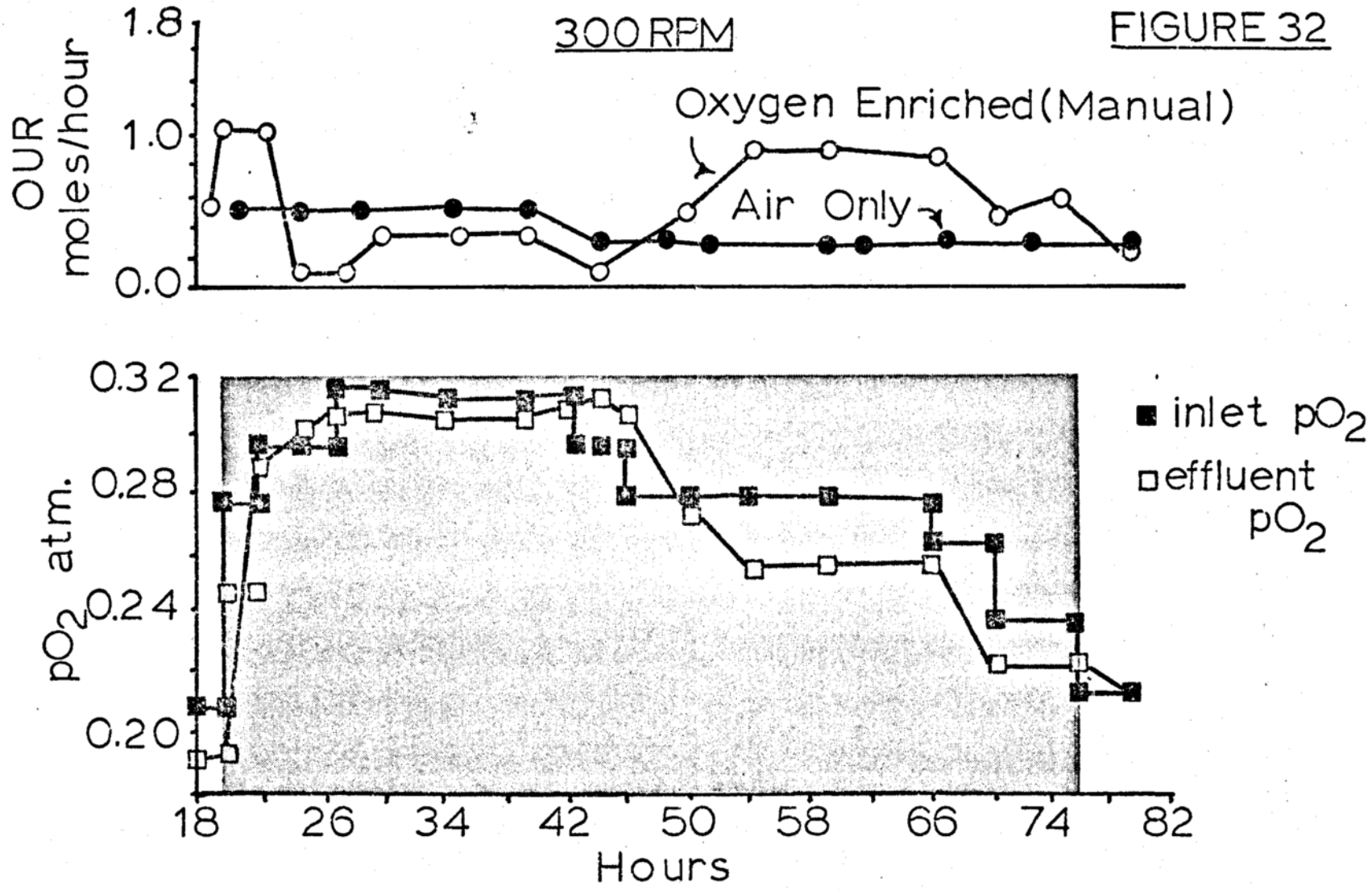
e. Effect of the Method of Addition of Oxygen on the Amount of Oxygen Taken Up by the Culture

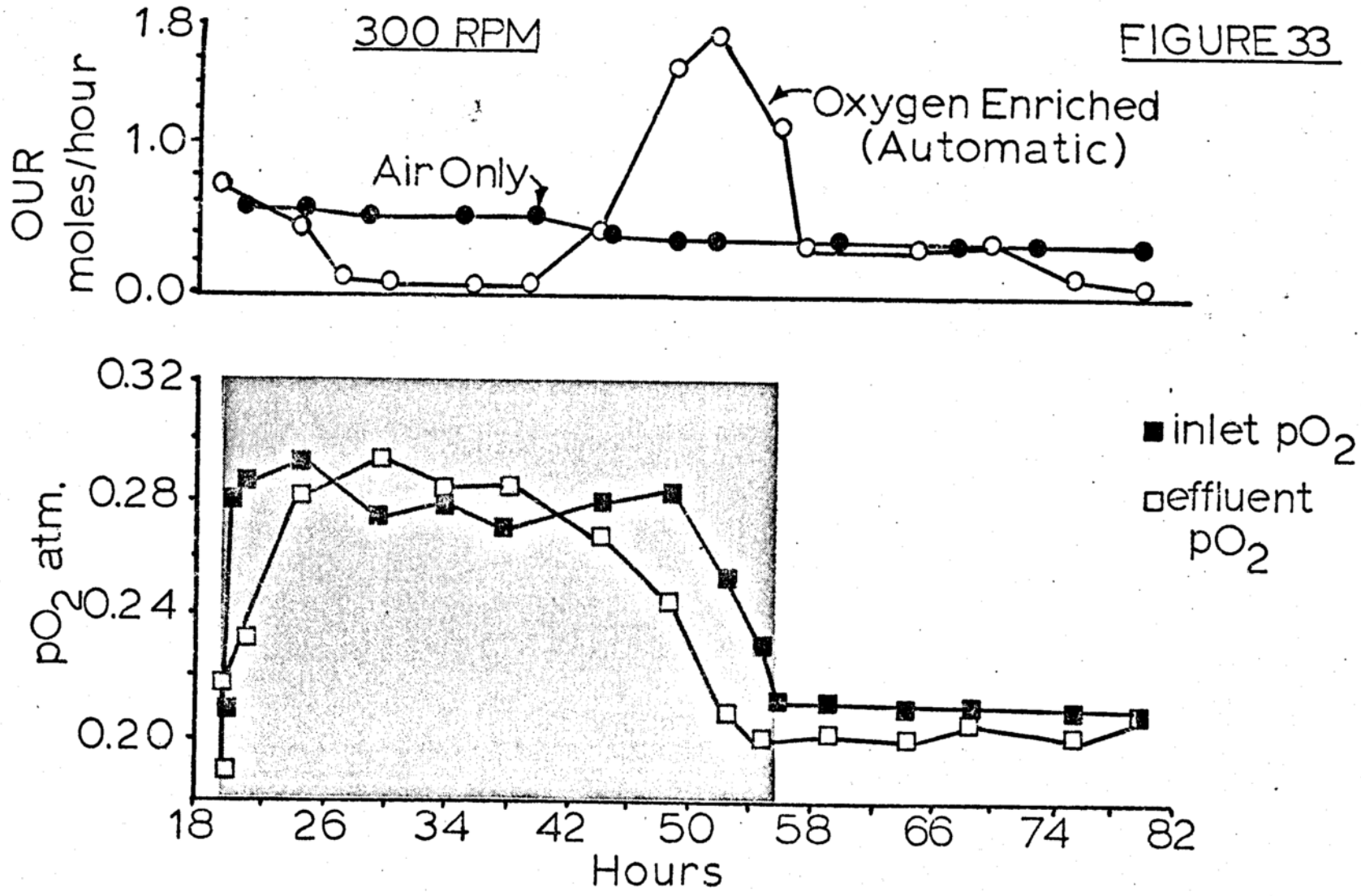
The oxygen uptake rates were calculated for the neomycin fermentations as 300 rpm and 400 rpm impeller speed. These results are presented in figures 32, 33, 34, and 35 and summarized on tables XII and XIII. Figures 32 and 34 show the results when the manual method of enrichment is used. Figures 33 and 35 are the results using the automatic enrichment method.

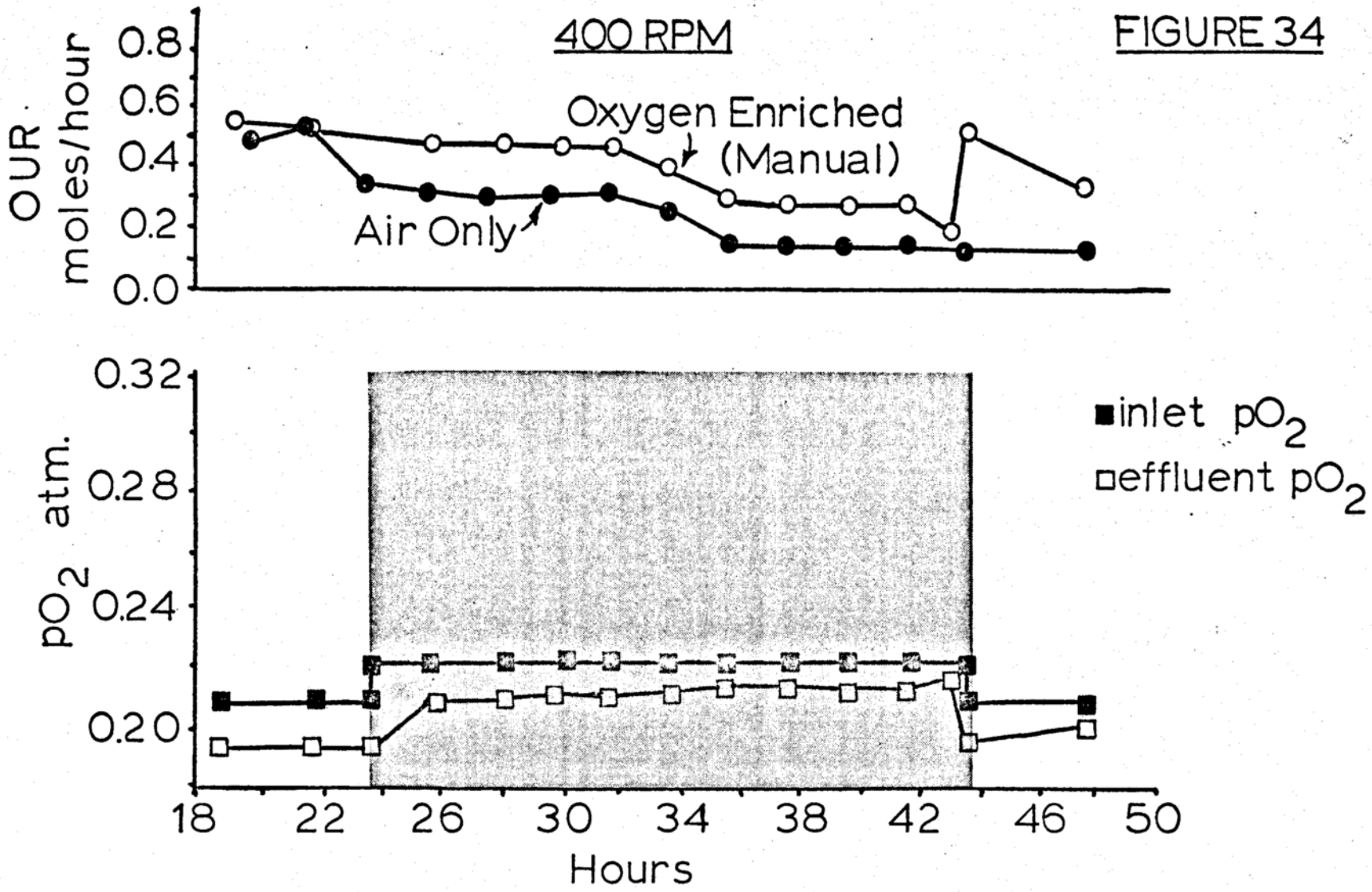
The OUR data from figure 32 suggests that during the first half of the period of manual O_2 enrichment the culture is undergoing some sort of adjustment to growth under elevated pO_2 levels. The very low OUR values are below that able to be measured.

A similar behavior showing suppressed initial OUR is seen in figures 33 and 35. Both of these fermentations utilized the automatic method of oxygen addition. The OUR values from these fermentations remain below the OUR values of the air fermentations for approximately 60 to 70 percent of the duration of O_2 enrichment.

Curiously, the data from figure 34 at 400 rpm using manual O_2 addition does not show this initial period of suppressed OUR. Only a small elevation in the inlet pO_2 (0.012 atms.) was required to maintain the dissolved oxygen tension at a level of 0.05 atm. during this fermentation. This small change in inlet pO_2 apparently did not result in suppression of the OUR of the culture. This would suggest that the reason for the OUR behavior in figures 32,


300 RPM
FIGURE 32
OUR moles/hour
1.8
1.0
0.0
Oxygen Enriched (Manual)
Air Only
0.32
0.28
0.24
0.20
pO_2 atm.
inlet pO_2
effluent pO_2
18
26
34
42
50
58
66
74
82
Hours


300 RPM
FIGURE 33
OUR
moles/hour
1.8
1.0
0.0
Air Only
Oxygen Enriched
(Automatic)
0.32
0.28
0.24
0.20
pO_2 atm.
inlet pO_2
effluent
pO_2
18
26
34
42
50
58
66
74
82
Hours


400 RPM
FIGURE 34
OUR moles/hour
0.8
0.6
0.4
0.2
0.0
Oxygen Enriched
(Manual)
Air Only
pO_2 atm.
0.32
0.28
0.24
0.20
inlet pO_2
effluent pO_2
18
22
26
30
34
38
42
46
50
Hours

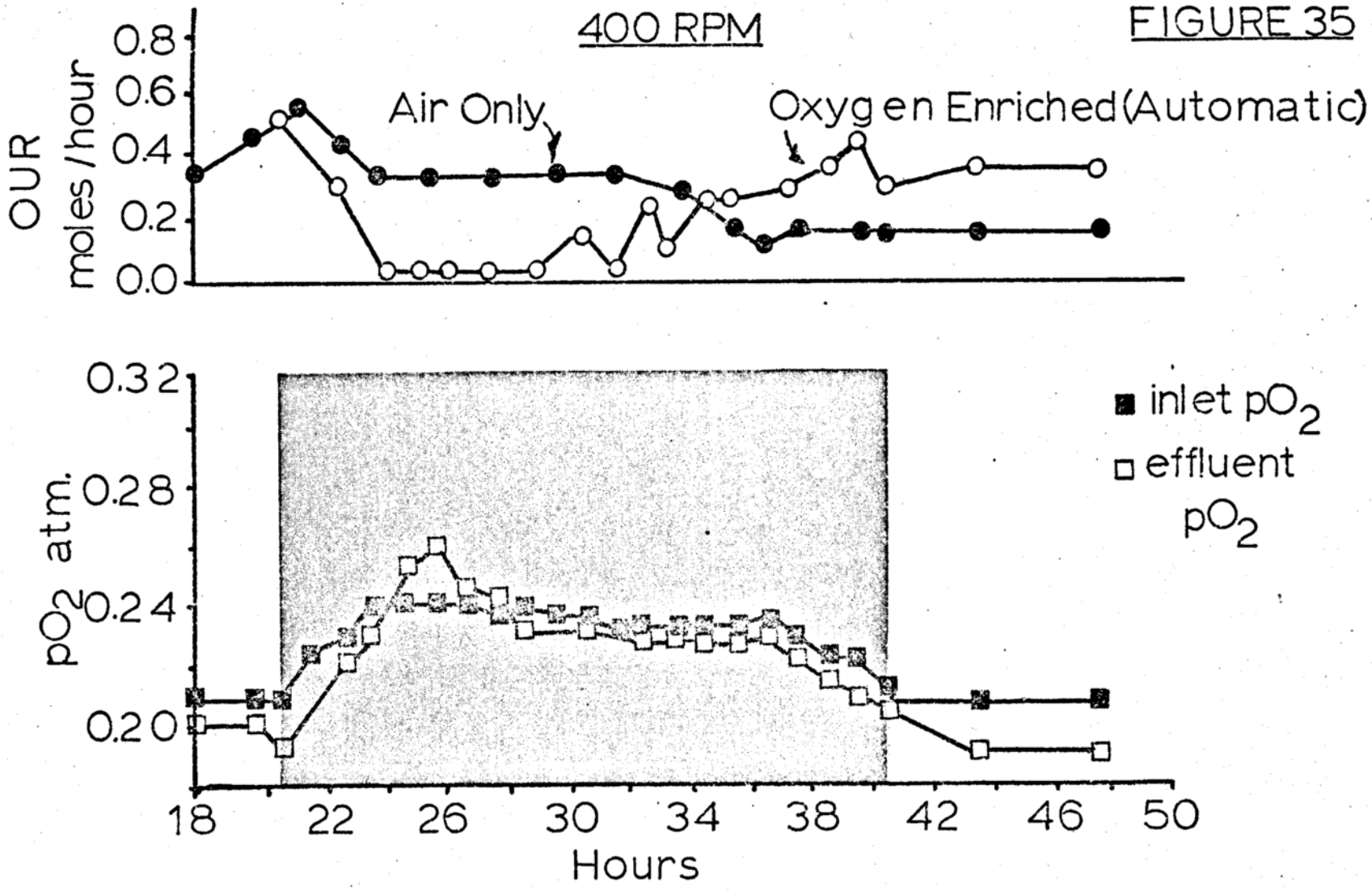

400 RPM
FIGURE 35
OUR
moles/hour
0.8
0.6
0.4
0.2
0.0
Air Only
Oxygen Enriched (Automatic)
0.32
0.28
0.24
0.20
pO_2 atm.
inlet pO_2
effluent pO_2
18
22
26
30
34
38
42
46
50
Hours

Table XIII

SUMMARY OF THE INCREASED YIELD OF NEOMYCINS IN RESPONSE TO BOTH MANUAL AND AUTOMATIC METHODS OF OXYGEN-ENRICHED AERATION TO MAINTAIN 0.05 ATM. DISSOLVED OXYGEN

- 0.5 vvm initial aeration rate -

IMPELLER RPM	METHOD OF O_2 ENRICHMENT	ENRICHMENT TIME IN HOURS	PSIG O_2 USED	MOLES O_2 USED	YIELD OF NEOMYCIN IN GRAMS PER 25 L.	NEO.GAIN PER O_2 USED (1)	NEO.GAIN PER O_2 TAKEN UP (2)	% O_2 USED (3)
300	None	0	0	0	37.0	-	-	-
300	*Manual	55	1900	222	58.25	0.003	0.050	6
300	**Automatic	34	950	111	47.75	0.003	0.038	8
400	None	0	0	0	45.75	-	-	-
400	Manual	19	100	11.6	60.0	0.038	0.17	23
400	Automatic	19	200	23.3	60.75	0.020	0.67	3

*MANUAL OXYGEN ENRICHMENT: Continuous enrichment at relatively constant total aeration rate of 0.5 vvm to maintain 0.05 atm. dissolved oxygen tension. O_2 flow varied manually in response to demand.

**AUTOMATIC OXYGEN ENRICHMENT: O_2 added in shots at 5 liters/minute in response to oxygen demand to maintain 0.05 atm. dissolved oxygen tension. Fluxuations in D.O.T. less than $\pm$ 0.004 atm.

(1) Grams of Neomycin Gained per Gram of O_2 Sparged Through Fermenter

(2) Grams of Neomycin Gained per Gram of Enriched O_2 taken up by the Culture

(3) Percent of Enriched O_2 taken up by the culture determined from comparison of Oxygen Uptake Rate curves of identical fermentations with and without oxygen enrichment.

33, and 35 is due to the addition of too much oxygen too fast early in the period of oxygen-enrichment for this slow growing organism. Comparison of the manual and automatic addition data suggests that addition of O_2 in 'shots' increases the period of suppressed OUR. The manual method of stepwise elevation of the pO_2 results in much less OUR suppression early in the period of enrichment.

Table XIII summarizes the actual amount of enriched O_2 that resulted in an increase in the final yield of neomycin. In three of the four oxygen-enriched fermentations, less than ten percent of the added O_2 was actually taken up by the culture. The amount of O_2 utilized by the culture appears to depend on the method of O_2 addition. When the OUR was not suppressed, 23 percent of the added O_2 was utilized.

However, the final yield of neomycin does not appear to be influenced by the method of oxygen-enrichment. Both methods result in the same magnitude of increased neomycin yield per gram of oxygen taken up.

The approximate material balance for oxygen for the neomycin fermentations can be assumed to be:

$$O_{2\,(\text{total oxygen uptake})} = O_{2\,(\text{in cells})} + O_{2\,(\text{evolved } CO_2)}$$

Medium carbohydrate and antibiotic products can be cancelled from each side of the equation because they are approximately the same oxidation level. The quantity of oxygen evolved as dissolved CO_2 was not calculated because of the changing pH and because the CO_2 absorption coefficient was not determined for this medium (106). The percentage of oxygen taken up by the culture recovered as either

cell mass or CO_2 can be calculated using the total gas-phase CO_2 evolved, cell dry weight (assuming the oxygen content of the cells is 27 percent), and the total moles of oxygen taken up.

The quantity of cell mass produced from the S. fradiae fermentations at 300 rpm and 400 rpm was 3.5 to 4.0 grams per liter cell dry weight. Using this value, the average percentage of O_2 recovered for all of the air and air + O_2 fermentations was 76 percent with a range of 58 to 99 percent.

f. Effect of Oxygen-Enrichment on the Ratio of Neomycin B and C Produced

Neomycin produced by S. fradiae is composed of approximately five components of varying antimicrobial activity. Neomycin B, the major component, is the most active with antimicrobial activity decreasing in order of neomycin C to neamine. Mono-acetyl derivatives of neomycin B and C (LP_B and LP_C) are not active. Neomycin D, E, and F if present are usually in extremely low percentage so that they can be eliminated from the potency calculation.

The relative antimicrobial response of neomycin B (Upjohn ref. 7558-DEV-120A) and neomycin C (Upjohn U-16010 ref. 6846-HKJ-116) were determined for B. subtilis Marburg. The relative microbial response of neomycin C was determined to be 77 percent of the response of neomycin B. This value is some what higher than that reported for Staphylococcus aureus ATCC 6538P (30 to 36 percent), Klebsiella pneumoniae ATCC 10031 (33 to 40 percent) (61), and Staphylococcus epidermidis ATCC 12228 (50 percent) (62). The relative response of 77 percent was used in the method for correction of the bioassay data for fermentations containing

more than five percent neomycin C. (Calculation Section 7).

Fermentation harvest samples were preliminarily screened for the presence of neomycin C and neamine by paper chromatography. The ratio of neomycin B to C was quantitated by GLC.

Neomycin B, C, and neamine could be detected in all fermentations. No neomycin D, E, or F were able to be detected by paper chromatography or GLC methods. Neamine was present in only trace amounts and was not quantitated.

The effect of oxygen-enrichment on the ratio of neomycins produced is summarized in table XIV. Increasing impeller speed decreases the final percentage of neomycin C present in both air and air + O_2 fermentations. The percentage of neomycin C present in the fermentation after 100 hours also appears to be effected by oxygen-enrichment. The graph of the data from table XIV, figure 36, shows that the increase in total neomycins produced in response to oxygen-enrichment is due to an increase in the production of neomycin B, the desired product. (Since neomycin B is more active than neomycin C, it is desirable to produce as little C as possible during the fermentation.)

However, the pH behavior of this fermentation is influenced by O_2 enrichment at 100 rpm and 200 rpm impeller speeds. The final column of table XIV presents the 100 hour pH of each fermentation. This data suggests that a higher final pH may favor a decrease in the final percentage of neomycin C present. At 100 rpm and 200 rpm oxygen-enrichment appears to favorably effect the viability of the culture resulting in a higher final pH and thus less neomycin C.

The B/C ratio time course data for 200 rpm shown in figure 37

Table XIV

SUMMARY OF THE EFFECT OF OXYGEN ENRICHED AERATION ON THE RATIO OF NEOMYCIN B AND C PRODUCED BY S. fradiae AT VARIOUS IMPELLER SPEEDS

- 0.5 vvm aeration rate -

IMPELLER RPM	O_2 ENRICHMENT	CORRECTED# TOTAL YIELD GM/L.	B/C RATIO*	GRAMS/L. NEO. B+	GRAMS/L. NEO. C+	PERCENT NEO. C	FINAL pH
100	None	0.0428	0.36	0.0113	0.0315	74	5.1
100	Yes	0.196	3.5	0.152	0.0437	22	6.6
200	None	0.533	2.24	0.368	0.164	31	6.9
200	Yes	0.891	16.0	0.838	0.052	6	7.8
300	None	1.54	4.84	1.27	0.263	17	8.0
300	Yes	2.22	4.21	1.79	0.426	19	8.0
400	None	1.79	10.3	1.63	0.159	9	8.2
400	Yes	2.46	11.1	2.25	0.204	8	7.9

* B/C RATIO DETERMINED BY GLC METHOD

+ NEOMYCIN DETERMINED AS SULFATE 655 mg base/gm

CORRECTED BY METHOD USED FOR FERMENTATIONS CONTAINING MORE THAN 5 PERCENT NEOMYCIN C, ASSUMING RELATIVE RESPONSE OF NEOMYCIN C OF 77 PERCENT.

FIGURE 36

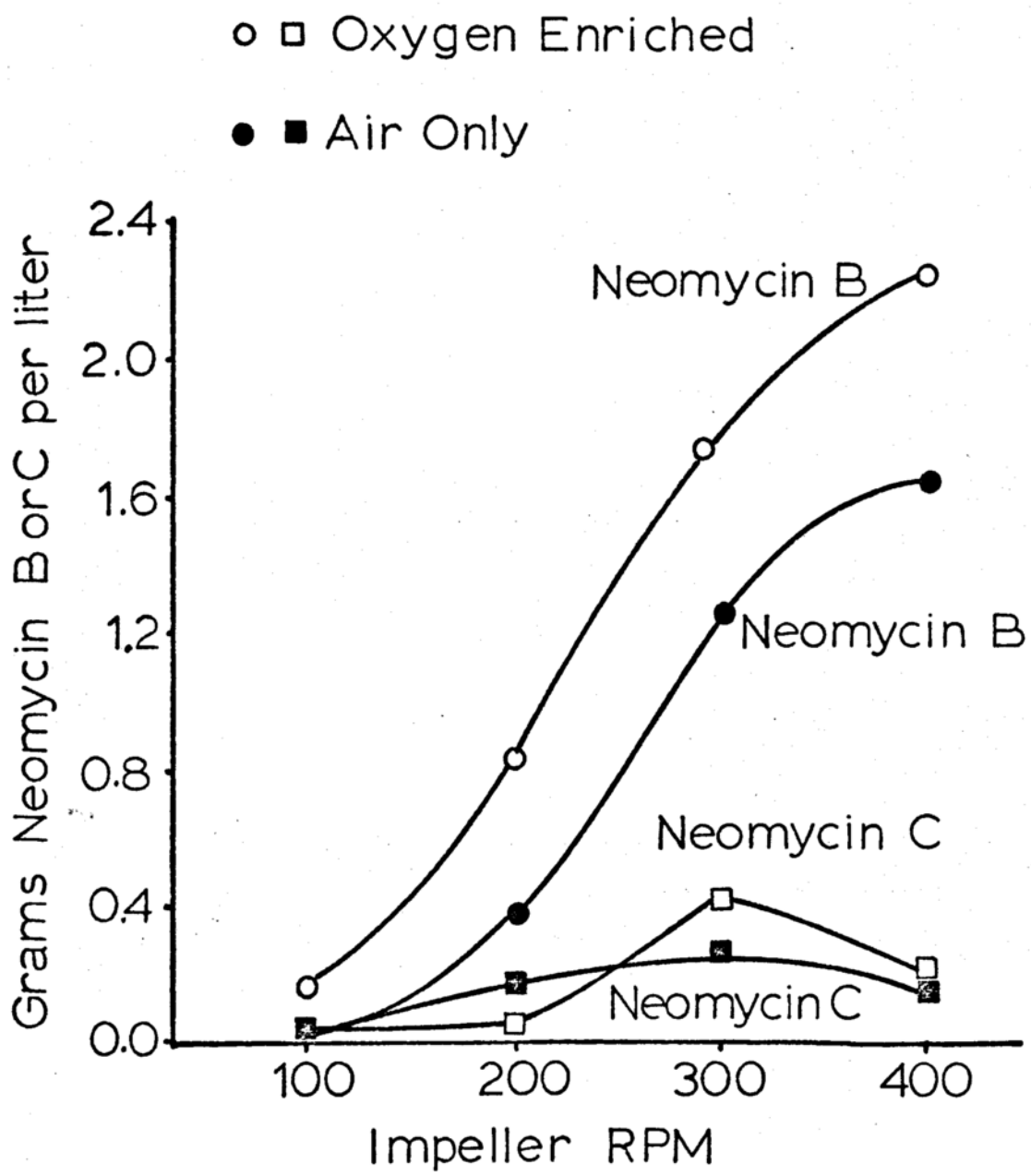
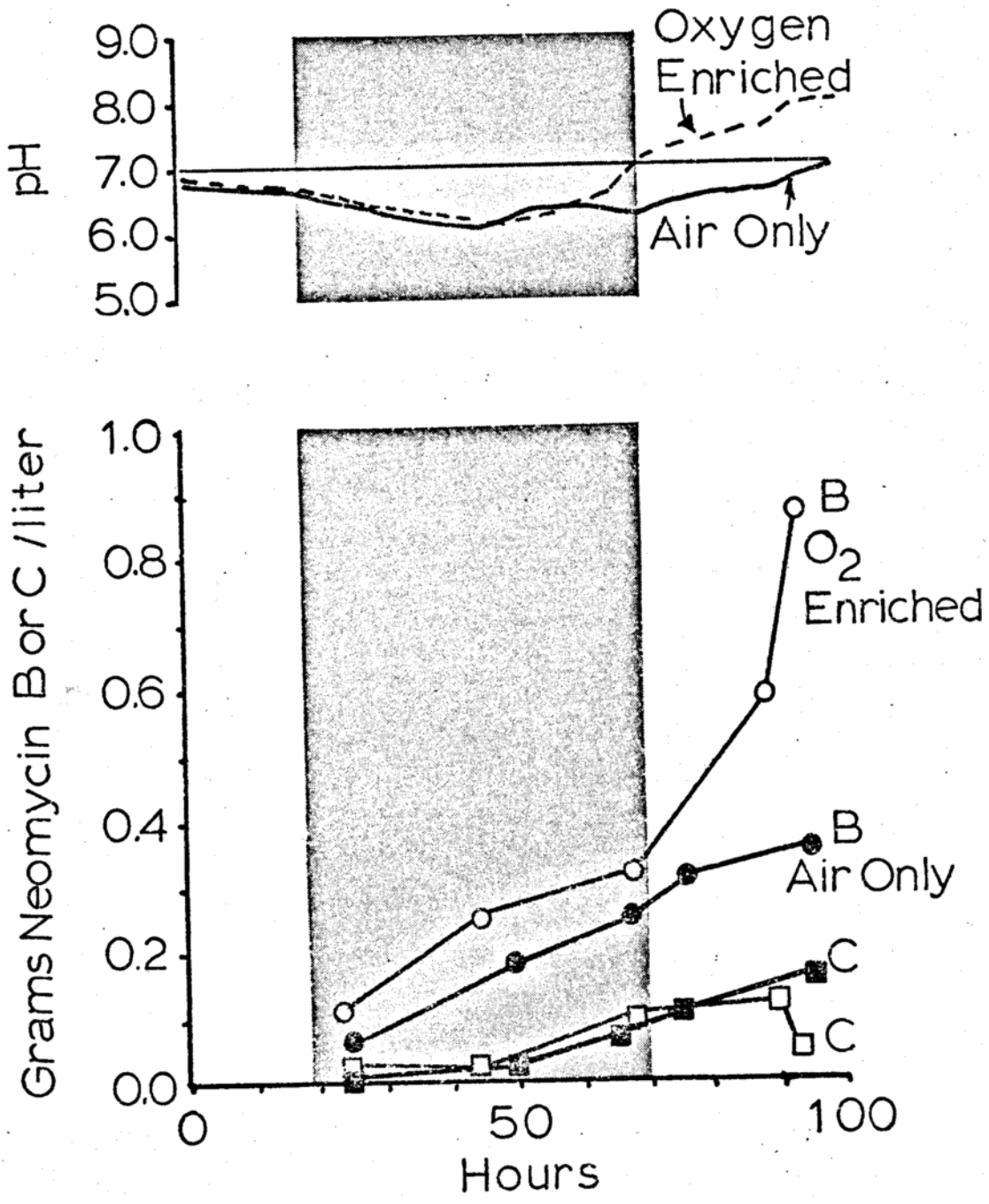

o □ Oxygen Enriched
● ■ Air Only
Grams Neomycin B or C per liter
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Neomycin B
Neomycin B
Neomycin C
Neomycin C
100
200
300
400
Impeller RPM

FIGURE 37

200 RPM



further suggests that when the pH of the O_2 enriched fermentation increases above pH 7, neomycin B was produced in preference to neomycin C.

At 300 rpm and 400 rpm the pH behavior of the fermentation is not affected by oxygen enrichment. Under these conditions, no decrease in the final percentage of neomycin C was observed.

Further evidence that the ratio of neomycin B and C is affected by pH is presented in figure 38. This figure shows a fermentation without oxygen-enrichment run under conditions of pH control at pH 7. With pH control, the amount of neomycin produced during the second half of the fermentation decreases to approximately zero. An identical fermentation without pH control contained 9 percent neomycin C after 100 hours of incubation.

D. Production of Bacitracin by Bacillus licheniformis ATCC 10716

1. Bacitracin: Chemistry, Biochemistry, and Commercial Production

The peptide antibiotic bacitracin produced by strains of B. licheniformis and B. subtilis consists of a group of closely related dodecapeptides containing cyclic structures. Similar peptide antibiotics are produced by many Bacillus species (94). At least ten bacitracins are known, but the principle antibacterial active component of the commercial product is bacitracin A. (figure 39) This structure includes a Δ-2 thiazoline ring and a cyclic peptide containing amino acids of alternating configurations and one $\alpha \rightarrow \epsilon$ peptide bond (104). Bacitracin A undergoes spontaneous conversion to the biologically inactive F form under alkaline conditions by deamination of the N-terminal isoleucyl moiety. Bacitracin B is believed to undergo a similar conversion to an inactive F form (95). Some of the bacitracins that can be separated

FIGURE 38

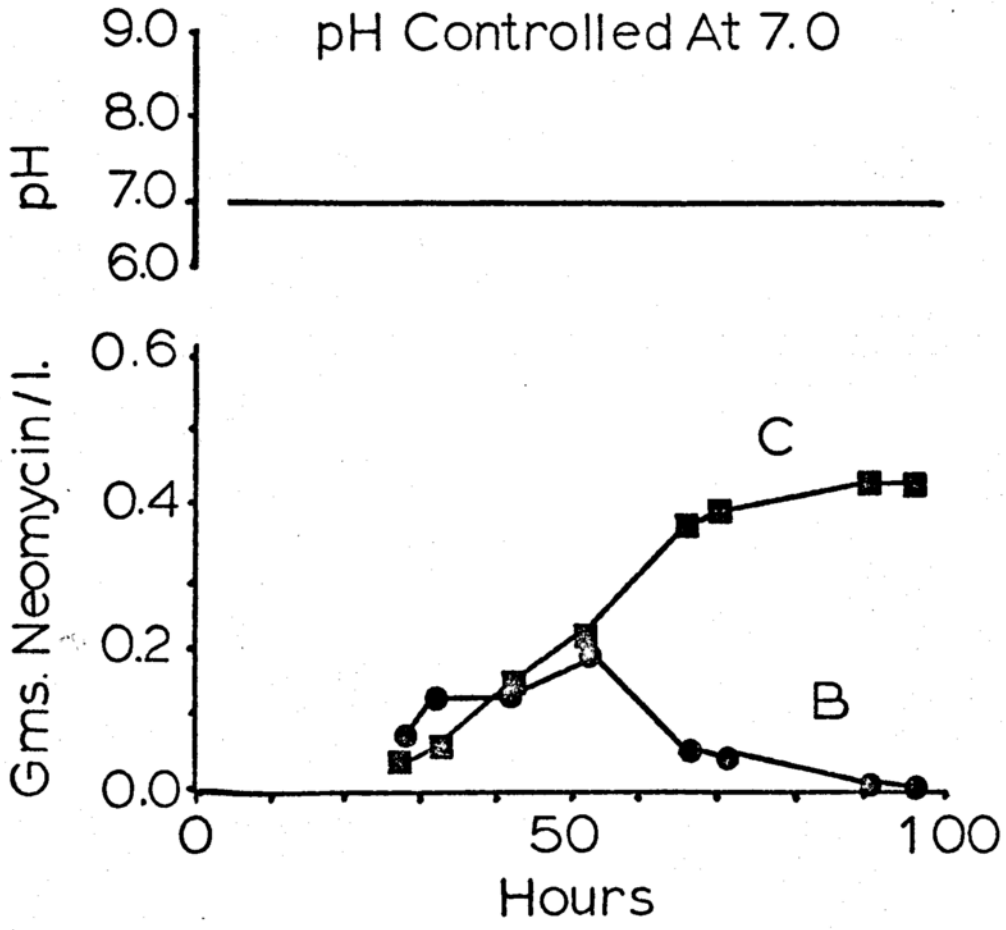
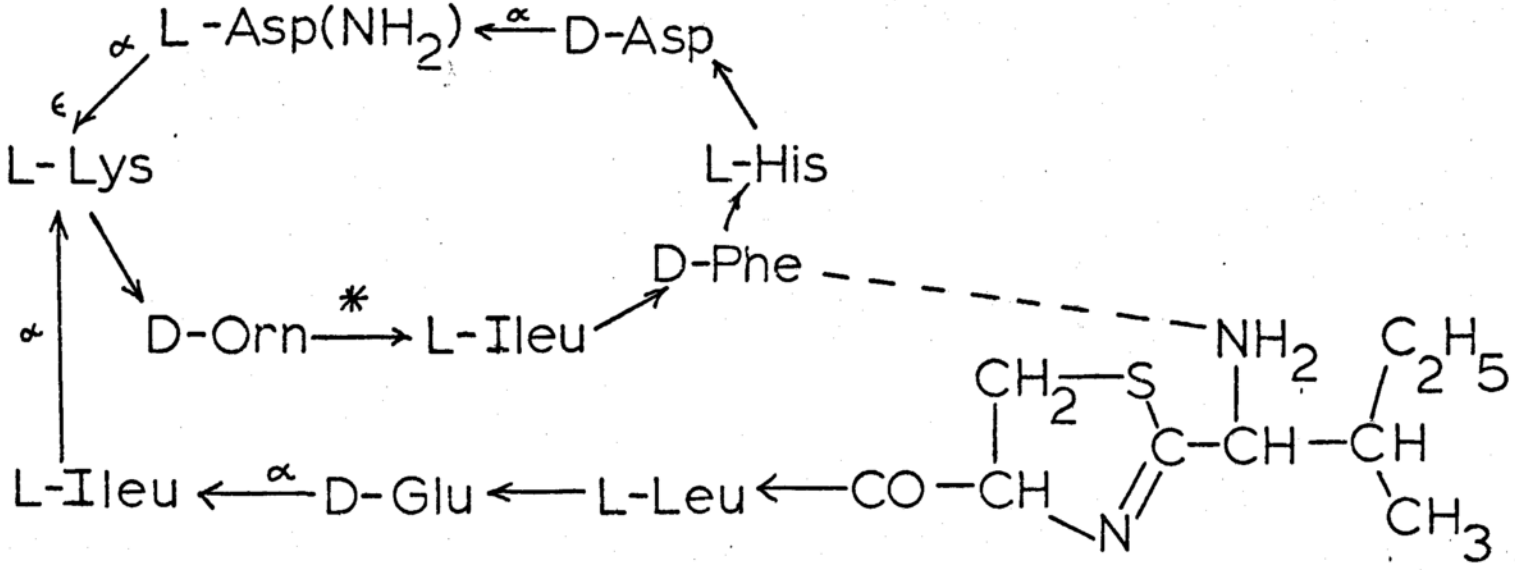

pH Controlled At 7.0
9.0
8.0
7.0
6.0
pH
0.6
0.4
0.2
0.0
Gms. Neomycin/l.
C
B
0
50
100
Hours

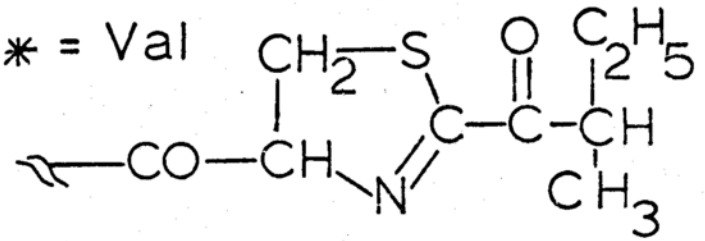
FIGURE 39



Bacitracin A

Bacitracin B * = Val

Bacitracin F



by counter current liquid chromatography are believed to be formed by transformation of unidentified peptides previously released into the medium. Bacitracins A and B, however, appear to be direct biosynthetic products. (95)

Bacitracin A has potent antimicrobial activity against gram positive bacteria. Bacitracins B and C have 87 percent and 30 percent of the activity of A respectively (53). Bacitracin F is not active while the relative activity of D, E, and G are very low. All bacitracins A, B, C, and F have potent nephrotoxicity which limits their systemic use in humans (95).

Bacitracin inhibits cell wall synthesis in sensitive organisms by interrupting the transport of cell wall components. Specifically, it prevents the dephosphorylation of a C_{55} pyrophosphate carrier of disaccharide wall units and thus the recycling of this lipoidal material (96). The antibiotic has also been reported to inhibit protein synthesis, function as an antimembrane agent (97), and to bind divalent metals (98).

Conditions for the production of this antibiotic in submerged culture are essentially the same as those for sporulation. The antibiotic appears to be released into the medium beginning at the end of or shortly after exponential growth. Studies on the production of bacitracin in defined media have demonstrated that manganese ions are required for sporulation and bacitracin production (94). Although antibiotic production appears to be intimately associated with the sporulation process (94), Haavik (50) has recently presented evidence on a bacitracin-negative mutant of B. licheniformis which is able to sporulate. Optimal conditions for antibiotic formation have also been reported which repress sporulation (43).

Considerable study of the nutritional requirements for the production of bacitracin has been done in defined media. (43) The specific amino acids (and their configuration) which promote antibiotic formation have been examined using spheroplasts of B. licheniformis (94). The effect of catabolite repression by organic acids (99), the effect of inorganic phosphate(100), and the inhibitory effect of glucose due to low pH created by its metabolism have been examined. (101)

Commercially bacitracin is produced on complex media by strains of B. subtilis or B. licheniformis. Information on the commercial process can be obtained from a few production patents and reviews (102,103,43). The primary requirement for a production medium producing high titers is a five to seven percent concentration of a plant protein such as soy bean meal, flour, or grits. A small amount of carbohydrate (one to three precent) is required with the soy protein to prevent excess deamination of the proteins and early development of a high pH. Calcium carbonate is added to the medium when glucose or sucrose is utilized as the carbon source in order to buffer the medium against the early development of a low pH resulting in reduced final titers. Maltose is not utilized for bacitracin production(104). Ziffer (102) has stated that the presence of ammonium sulfate and another source of utilizable nitrogen can improve the production of a bacitracin producing strain of B. subtilis. Conditions of medium composition, pH and aeration level have been examined and shown to affect the final titer of bacitracin in production media (103,104,43).

2. Choice of Production Medium

Soy products for the bacitracin production medium were evaluated in cultures grown in 200 ml. of medium in 2000 ml. Erlenmeyer flasks

incubated at 37°C on a 300 rpm rotary shaker. Bacitracin titer was assayed by bioassay after 30 hours of incubation. The production medium of Ziffer(102) consisting of 50 gms./liter soy grits (ADM 63-380), 12 gms./liter sucrose or glucose, 2 gms./liter ammonium sulfate, and 2 gms./liter calcium carbonate supported the best titers of antibiotic in this shake flask system, 70 units/ml. Other production media (43) with varying concentrations of soybean meal, flour or grits, starch, dextrin, and calcium carbonate only supported the production of 30 units/ml. of antibiotic in this system using B. licheniformis ATCC 10716 as the producing organism.

A bacitracin producing strain of B. subtilis, strain ATCC 14593, was evaluated in the various production media. This strain produced the same level of antibiotic as B. licheniformis ATCC 10716 in the shake flask system. However when tested in 25 liter batch fermentations on the Ziffer medium, less than 10 units per ml.were produced by this B. subtilis strain. B. licheniformis ATCC 10716 was therefore chosen as the organism for further study.

Foaming of the soy grits medium both in shaken flasks and in 25 liter fermentations was extensive. Eight different silicone antifoams were evaluated for effectiveness on the production medium in the shake flask system. Each antifoam was added to the medium at concentration of 20 ppm, 50 ppm, and 100 ppm before autoclaving. Foam, percentage of cells sporulating, and antibiotic titer were assayed after 48 hours of incubation at 37°C. SAG 5440 (Union Carbide Co.) silicone antifoam was the most effective in controlling foaming at 20 ppm to 50 ppm without decreasing the percentage of cells sporulating yielding a three fold increase in titer over identical flasks without added antifoam.

3. Effect of Oxygen-Enriched Aeration on Production of Bacitracin by Bacillus licheniformis ATCC 10716

The effect of oxygen-enrichment on the production of bacitracin was studied at three different levels of power input. Impeller speeds of 400 rpm, 300 rpm, and 200 rpm were used with an aeration rate of 1.0 vvm.

In contrast to the neomycin study, bacitracin production was investigated at three different levels of O_2 enrichment: 0.01 atm., 0.02 atm., and 0.05 atm. of constant dissolved oxygen tension. Compressed oxygen was added to the aeration line using the automatic controller beginning at the point in the fermentation where the dissolved oxygen tension reached the desired control level.

In the following figures the shaded areas denote the period of oxygen-enrichment at different levels of constant DOT 0.01 atm., 0.02 atm., or 0.05 atm.

a. Effect on B. licheniformis ATCC 10716

i. Maximum Growth Rate

Table XV and figure 40 summarize the effect of maintaining the dissolved oxygen tension constant (using oxygen-enriched air) on the maximum growth rate of B. licheniformis. Maximum growth rates were determined from cell dry weight determinations. The maximum growth rate for this organism with air sparge was determined to be 0.6 ± 0.05 hour $^{-1}$. At each impeller speed examined, increasing the level of oxygen-enrichment significantly decreased the maximum specific growth rate and resulting final cell yield. The final yield of cells was decreased from 10 to 40 percent when oxygen-enrichment was applied. The data presented in figure 40 suggest that as the driving force of oxygen transfer during the

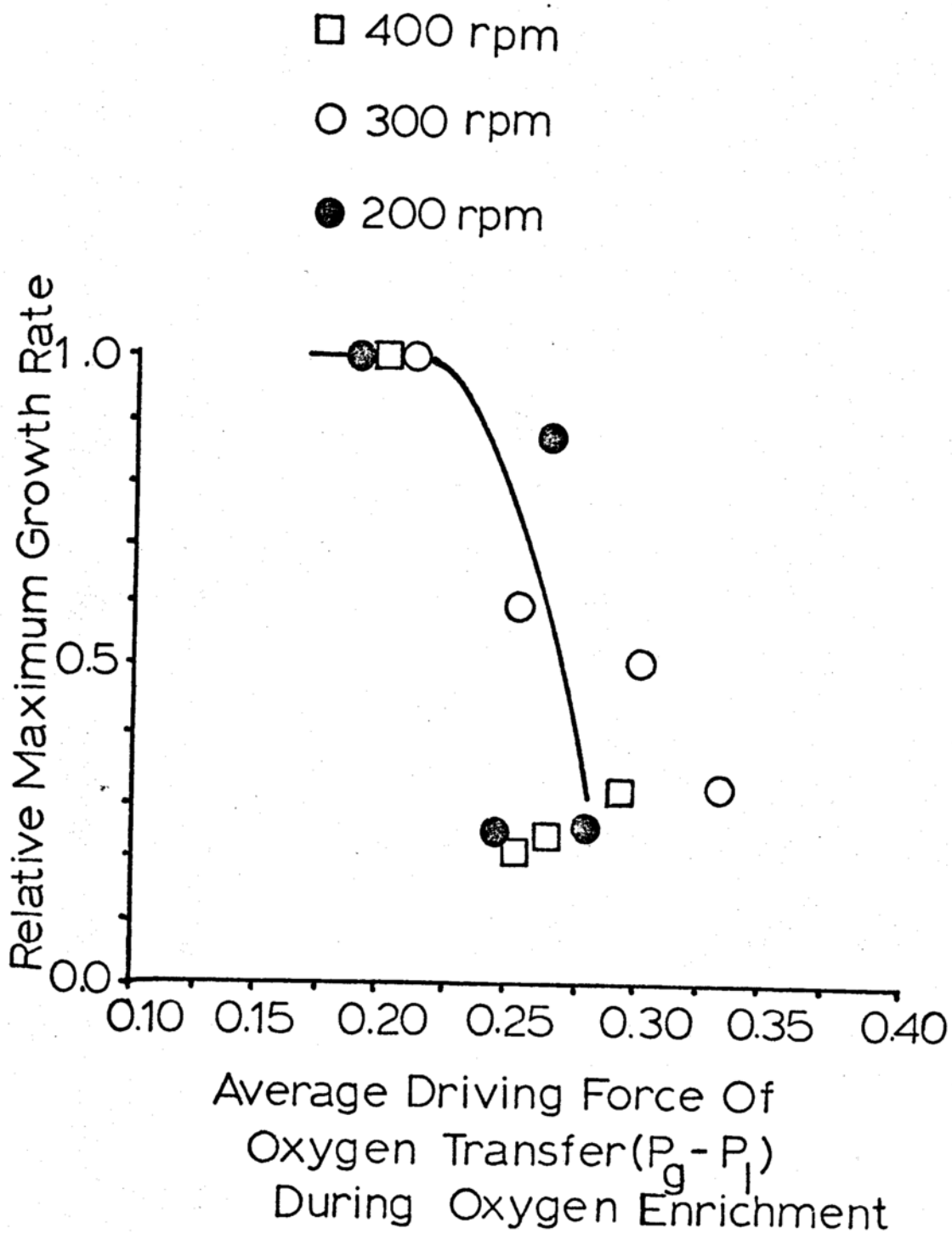
Table XV

EFFECT OF O_2 ENRICHMENT ON THE GROWTH OF *B. licheniformis*

IMPELLER SPEED	LEVEL OF O_2 ENRICHMENT (atm.)	DURATION OF ENRICHMENT	FINAL CELL YIELD[+]	MAXIMUM SPECIFIC GROWTH RATE ($hr.^{-1}$)
200	None	-	2.7	0.59
200	0.01	36.5 hr.	2.1	0.52
200	0.02	36.0	2.1	0.14
200	0.05	36.0	1.8	0.15
300	None	-	2.6	0.48
300	0.01	20.8	3.1	0.29
300	0.02	20.3	2.1	0.25
300	0.05	19.3	2.8	0.15
400	None	-	2.5	0.65
400	0.01	13.5	2.3	0.20
400	0.02	14.3	2.4	0.15
400	0.05	14.8	2.2	0.14

[+]approximate grams cell dry weight per liter

FIGURE 40


□ 400 rpm
○ 300 rpm
● 200 rpm
Relative Maximum Growth Rate
1.0
0.5
0.0
0.10
0.15
0.20
0.25
0.30
0.35
0.40
Average Driving Force Of
Oxygen Transfer($P_g - P_l$)
During Oxygen Enrichment

period of oxygen-enrichment is increased, the maximum growth rate of B. licheniformis decreases. If the average driving force (P_g-P_l) is increased by 50 percent during the period of enrichment, the maximum growth rate decreases to between 20 and 50 percent of its value when the organism is grown in air without dissolved oxygen control. This effect is most pronounced at higher oxygen transfer conditions, 400 rpm, where even DOT control at 0.01 atms. decreases the maximum growth rate to 30 percent of its value in air.

ii. Respiration

Oxygen-enrichment also strongly affects cellular respiration as monitored by the gas-phase evolution of carbon dioxide during the fermentation. Table XVI shows that increasing the relative oxygen transfer rate by a factor of approximately two by increasing the impeller speed from 200 to 400 rpm increases the total moles of CO_2 produced by the culture while the final cell yield is not significantly increased (table XV).

Increasing the driving force of oxygen transfer by O_2 enrichment results in increased CO_2 production. Figure 41 presents the CO_2 evolution data during the period of oxygen-enrichment as related to the average driving force of oxygen transfer during that period. At 200 and 300 rpm impeller speeds, if the driving force of oxygen transfer is doubled, the culture will produce 2.6 times the CO_2 (gas-phase). However, there appears to be an upper limit to this effect between 300 and 400 rpm. At 400 rpm impeller speed doubling of the driving force of oxygen transfer results in only 1.25 fold increase in CO_2 evolution.

The CO_2 evolution rate during the course of this fermentation

Table XVI

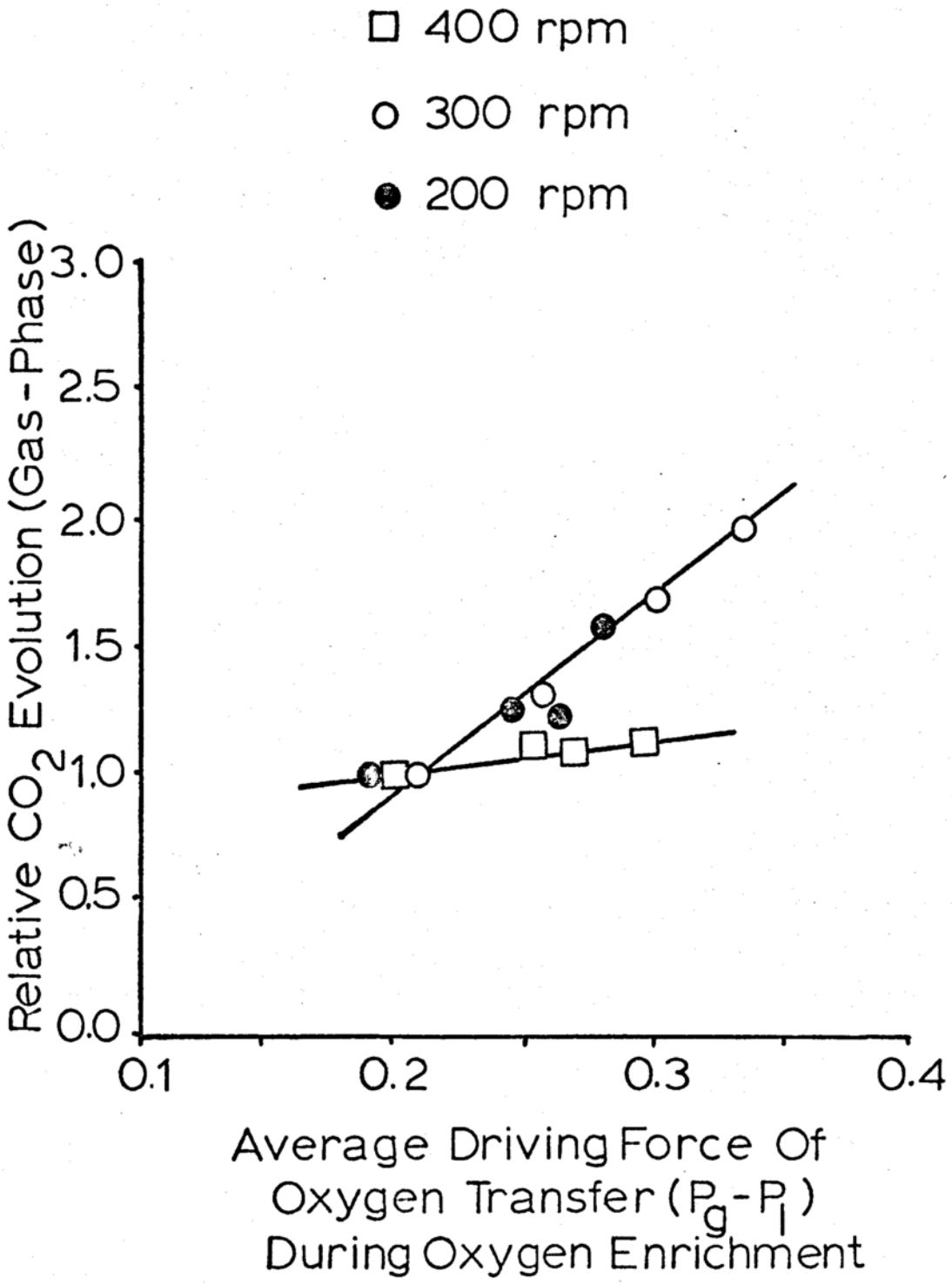
EFFECT OF OXYGEN-ENRICHED AERATION ON THE PRODUCTION OF CO_2 BY *B. licheniformis* AT VARIOUS LEVELS OF DOT CONTROL

IMPELLER RPM	O_2 LEVEL OF ENRICHMENT (atm.)	TOTAL MOLES CO_2 EVOLVED (GAS-PHASE)	MOLES CO_2 EVOLVED DURING ENRICHMENT	MOLES CO_2 DURING EQUIVALENT PERIOD (1)	RELATIVE CO_2	AVERAGE O_2 DRIVING FORCE (2)
200	None	11.5	-	-	1.0	0.188 atm.
200	0.01	14.0	11.7	9.5	1.23	0.265
200	0.02	14.2	12.6	10.1	1.25	0.246
200	0.05	17.7	16.5	10.5	1.57	0.282
300	None	20.5	-	-	1.0	0.21
300	0.01	21.6	16.7	12.6	1.32	0.258
300	0.02	27.5	23.0	13.7	1.68	0.302
300	0.05	29.6	25.3	12.8	1.97	0.336
400	None	33.5	-	-	1.0	0.201
400	0.01	30.4	17.7	15.6	1.13	0.294
400	0.02	30.3	21.8	20.5	1.07	0.268
400	0.05	32.2	25.1	22.3	1.13	0.254

(1) Moles CO_2 during an equivalent period during an identical fermentation using air sparge.

(2) Average driving force of oxygen transfer during the period of oxygen-enrichment (or equivalent period during unenriched fermentations). This is the average $(P_g - P_l)$ value for each hour of enrichment averaged over the period of time of enrichment.

FIGURE 41


□ 400 rpm
○ 300 rpm
● 200 rpm
Relative CO_2 Evolution (Gas-Phase)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.1
0.2
0.3
0.4
Average Driving Force Of
Oxygen Transfer ($P_g - P_l$)
During Oxygen Enrichment

is shown in figures 42, 43, and 44 for 200 rpm, 300 rpm, and 400 rpm respectively. In all cases, O_2 enrichment not only increased the peak carbon dioxide evolution value but the culture continued to evolve CO_2 at a two to five fold higher rate throughout the period of O_2 enrichment. After enrichment ceased, the CO_2 evolution rate fell significantly below that of the air fermentation.

The respiratory quotient during the period of oxygen-enrichment was somewhat depressed in relation to the R.Q. when sparging with air only. Respiratory quotient values of 1.4 ± 0.3 were observed using air sparge while values of 0.6 ± 0.1 to 0.9 ± 0.1 were calculated for the air + O_2 fermentations during the period of enrichment.

iii. Sporulation

Another index of the effect of oxygen-enriched aeration on the general physiology of this culture is the rate and extent of sporulation. Figures 45, 46, and 47 show that the final percentage of cells sporulating (air sparge) was increased from 80 percent to essentially 100 percent by increasing the impeller speed from 200 to 300 rpm.

In general, O_2 enrichment induced B. licheniformis to begin sporulating two to four hours earlier than the 'air' fermentations. This effect was observed at all levels of enrichment. The final percentage of sporulating cells, and the rate of sporulation did not appear to be affected by O_2 enrichment. The final titers of antibiotic produced (section 3b) do not appear to be directly related to the extent or rate of sporulation of this culture.

iv. pH Behavior

The 'pH profile' of the oxygen-enriched fermentations were

FIGURE 42

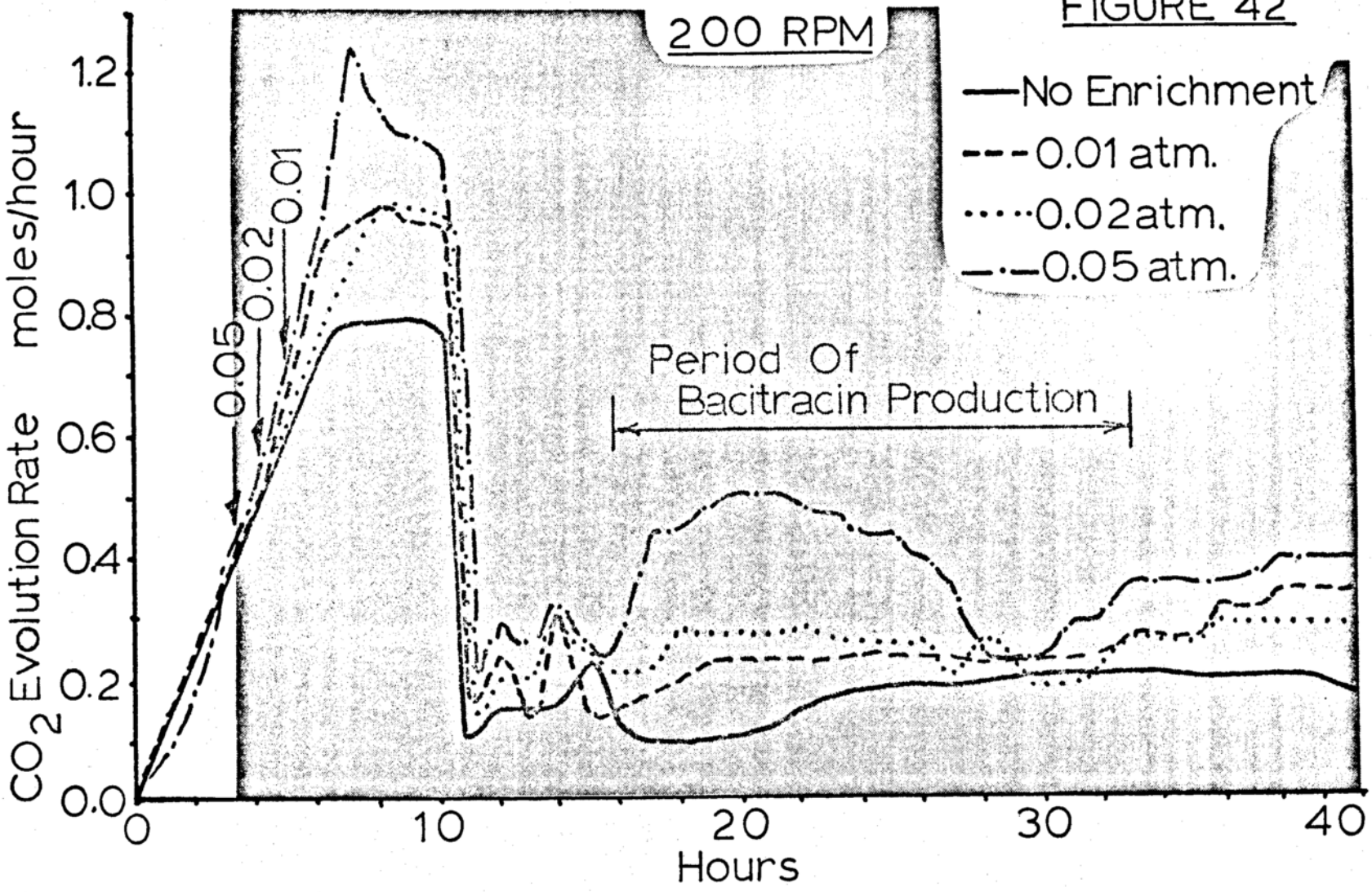

200 RPM
No Enrichment
0.01 atm.
0.02 atm.
0.05 atm.
0.05
0.02
0.01
Period Of
Bacitracin Production
CO_2 Evolution Rate moles/hour
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
10
20
30
40
Hours

FIGURE 43

300 RPM

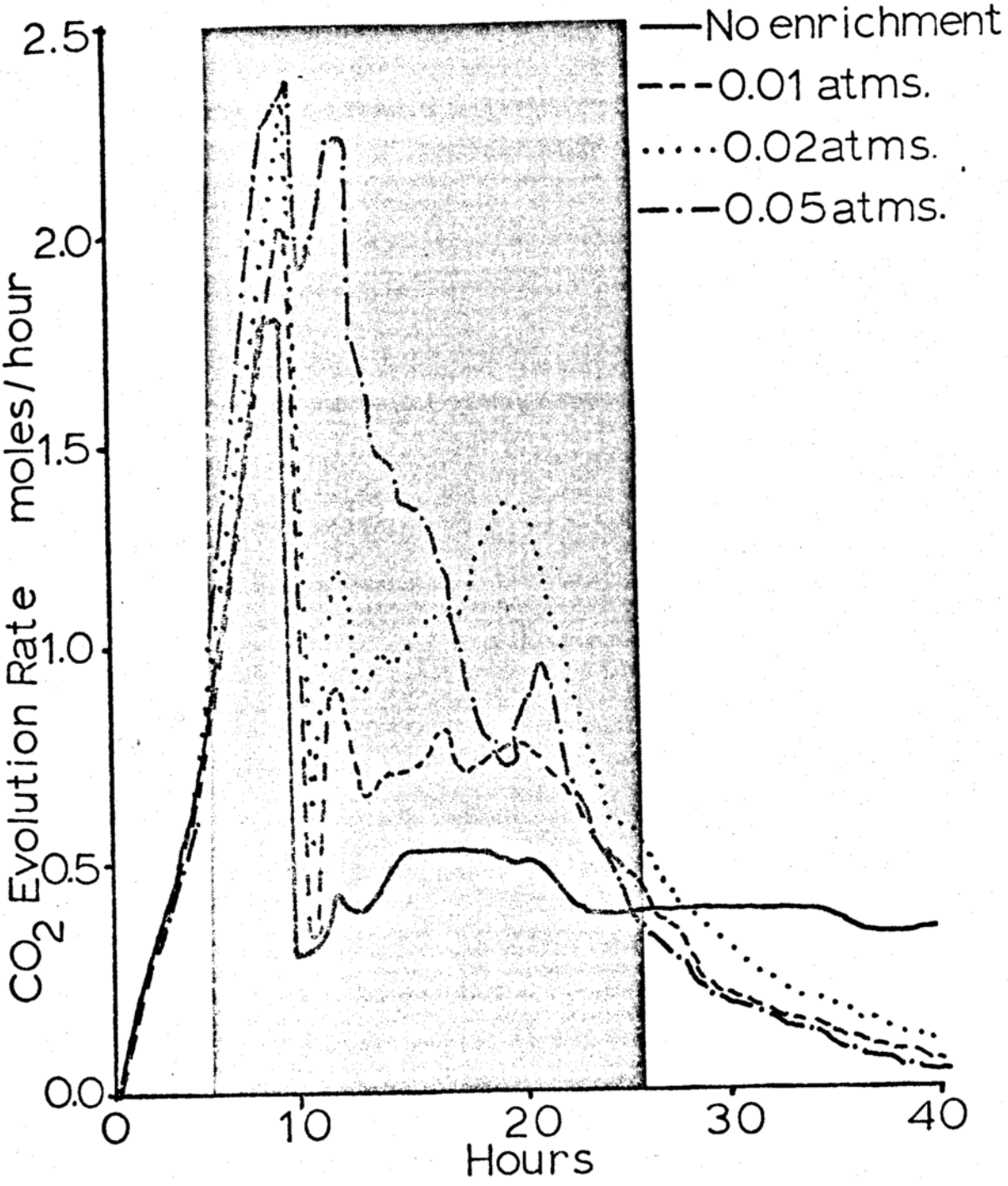


FIGURE 44

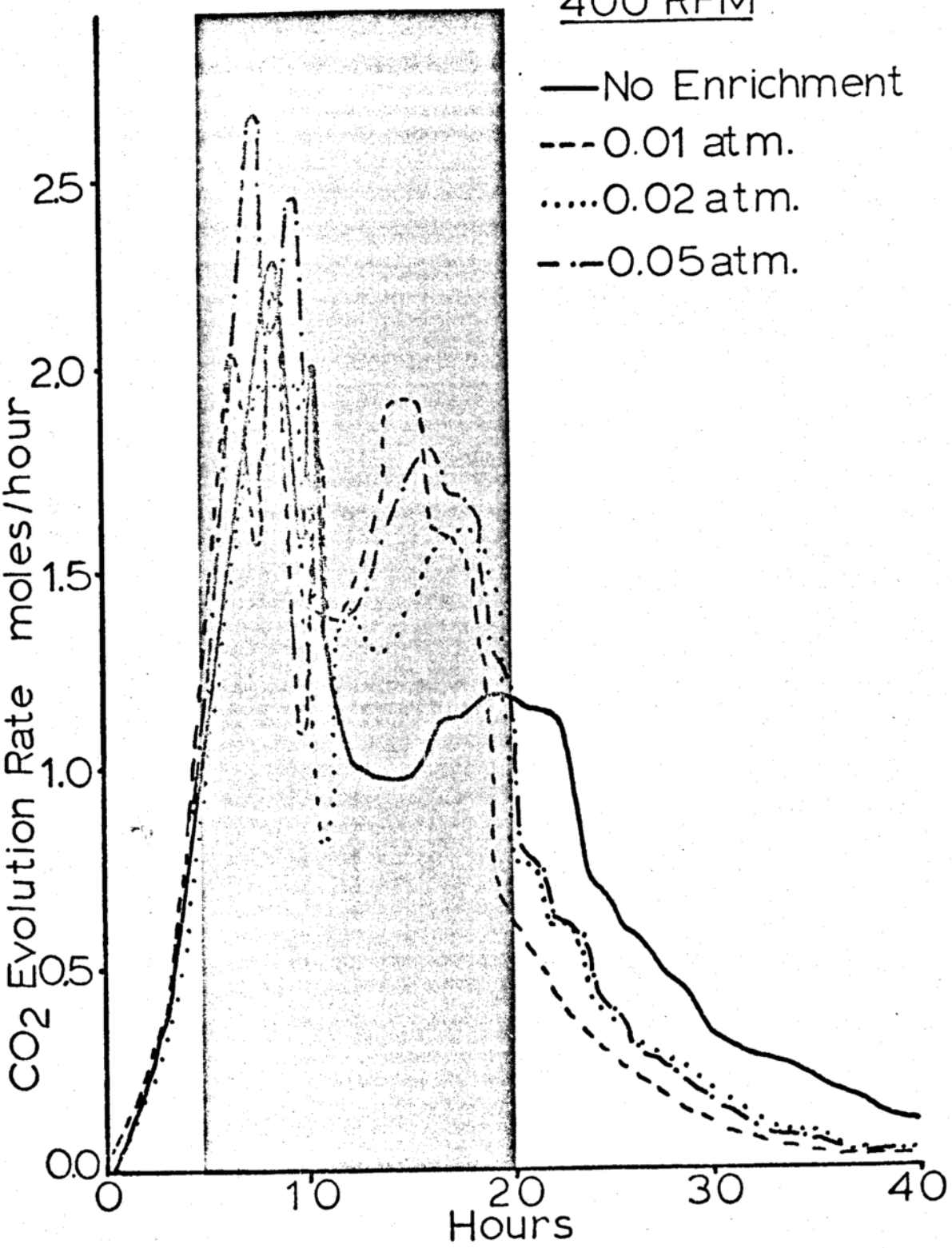

400 RPM
—No Enrichment
---0.01 atm.
.....0.02 atm.
-·-0.05 atm.
CO_2 Evolution Rate moles/hour
0.0
0.5
1.0
1.5
2.0
2.5
0
10
20
30
40
Hours

FIGURE 45

200 RPM

● No Enrichment

○ 0.01 atm.

□ 0.02 atm.

△ 0.05 atm.

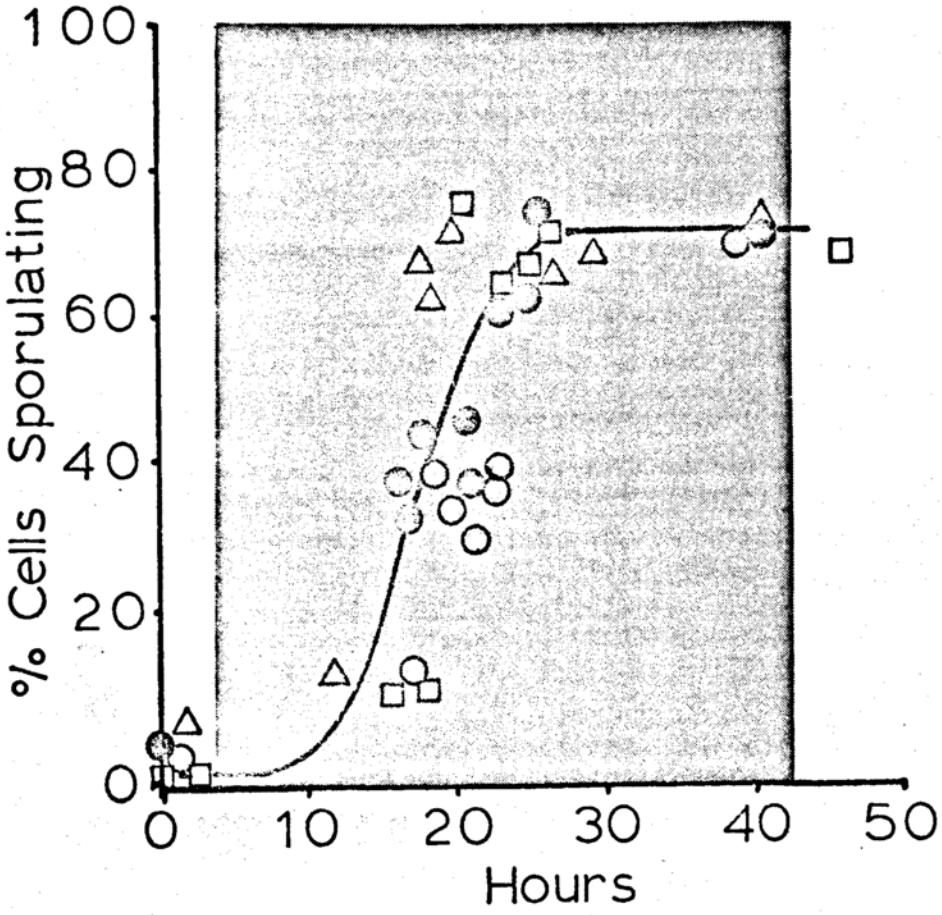


FIGURE 46

300RPM

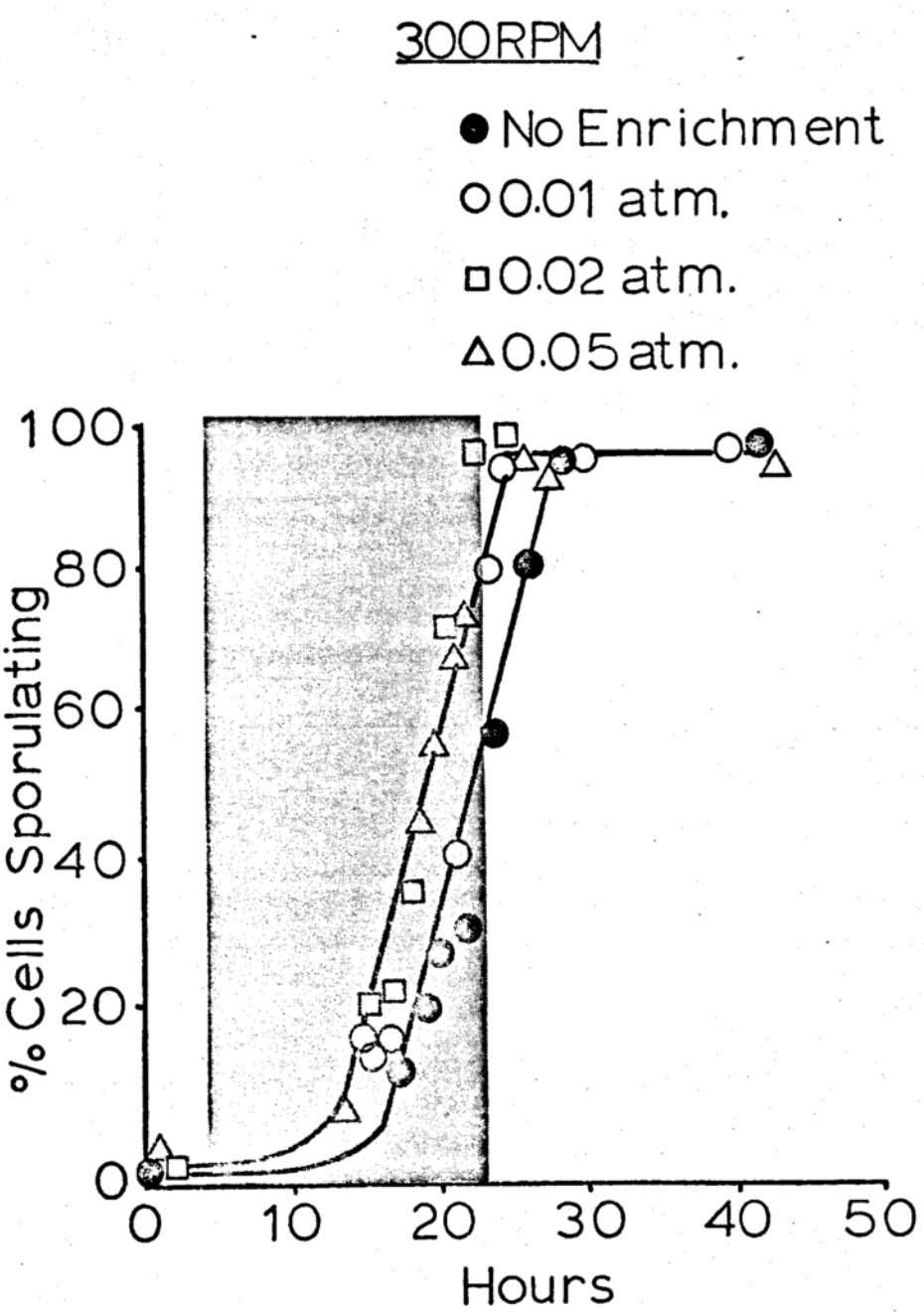
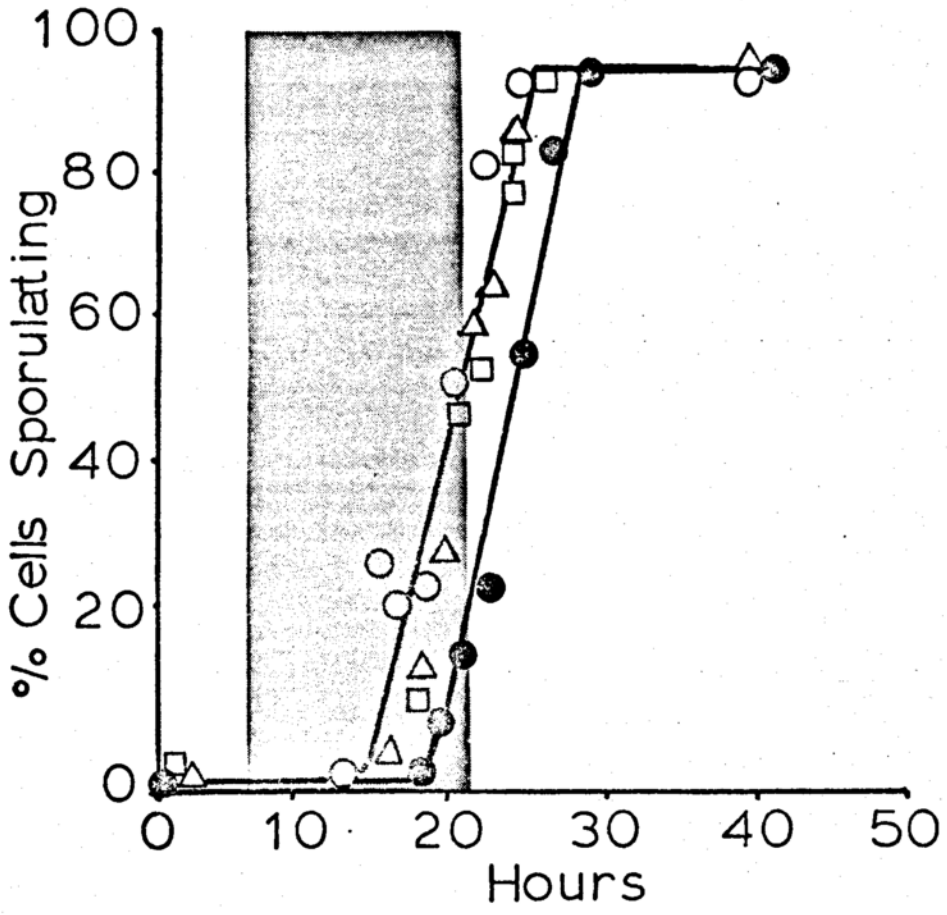


FIGURE 47

400 RPM

● No Enrichment
○ 0.01 atm.
□ 0.02 atm.
△ 0.05 atm.



significantly altered from that of the unenriched runs. (figure 48) As the level of enrichment was increased, the length of time until the period of increasing pH decreased. The rate of pH increase between 15 and 25 hours of incubation also appeared to be increased when O_2 enrichment was used. The final pH value of the fermentation however was not significantly changed.

b. Increasing Antibiotic Yield at Constant Impeller Speed

Table XVII and figure 49 contain the data demonstrating the effect of oxygen-enriched aeration on antibiotic production. At all three impeller speeds examined, the final titer of bioactive bacitracin produced was increased by O_2 enrichment. At the lower impeller speed, 200 rpm, the level of oxygen-enrichment did not correlate with the amount of increase in antibiotic. However, the data at 300 rpm and 400 rpm indicate that an optimum region of DOT control by O_2 enrichment may exist. As the impeller speed is increased from 300 to 400 rpm, enrichment at a level of 0.05 atm. appears to be the upper limit at which an increase in final titer is seen appears to be less than 0.01 atm. at 300 rpm and between 0.01 atm. and 0.02 atm. at 400 rpm. This data suggests that under conditions of constant power input with a specified medium, the region of DOT control by O_2 enrichment where an increase in titer can be realized may be narrow (0.04 - 0.05 atm.). It also suggests that this region may become more narrow under conditions of better O_2 transfer.

As the impeller speed was increased, better oxygen transfer conditions were obtained and the length of time O_2 enrichment decreased from 36 hours at 200 rpm to 14 hours at 400 rpm. Consequently, more antibiotic was gained per gram of enriched O_2 taken up by the culture as

FIGURE 48

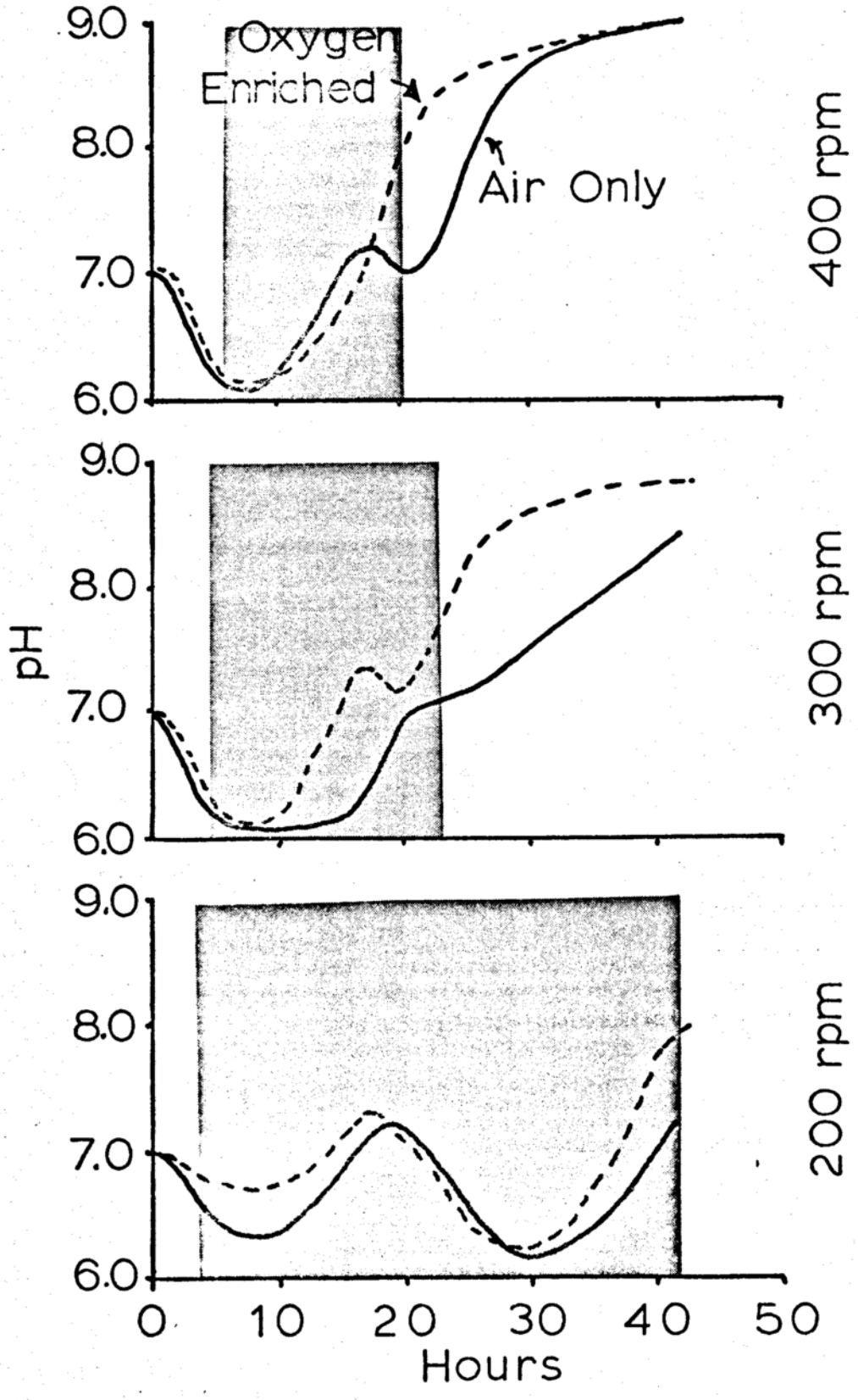

9.0
8.0
7.0
6.0
Oxygen
Enriched
Air Only
400 rpm
9.0
8.0
7.0
6.0
pH
300 rpm
9.0
8.0
7.0
6.0
200 rpm
0
10
20
30
40
50
Hours

Table XVII

SUMMARY OF THE INCREASED YIELD OF BACITRACIN IN RESPONSE TO OXYGEN-ENRICHED AERATION

IMPELLER RPM	LEVEL OF O_2 ENRICHMENT	MOLES O_2 USED	(1) BACITRACIN YIELD 25 L.	(2) BAC. GAIN PER O_2 USED	(3) BAC. GAIN PER O_2 UPTAKE	%(4) O_2 USED
200	None	-	60 gms.	-	-	-
200	0.01 atm.	224	129	0.0097	0.084	12
200	0.02	181	95	0.0061	0.085	7
200	0.05	423	72	0.0009	0.012	8
300	None	-	55	-	-	-
300	0.01	117	97	0.011	0.081	14
300	0.02	198	112	0.0091	0.45	2
300	0.05	279	194	0.016	0.20	8
400	None	-	87	-	-	-
400	0.01	126	90	0.0007	0.0053	14
400	0.02	121	161	0.019	0.16	12
400	0.05	127	201	0.028	0.16	18

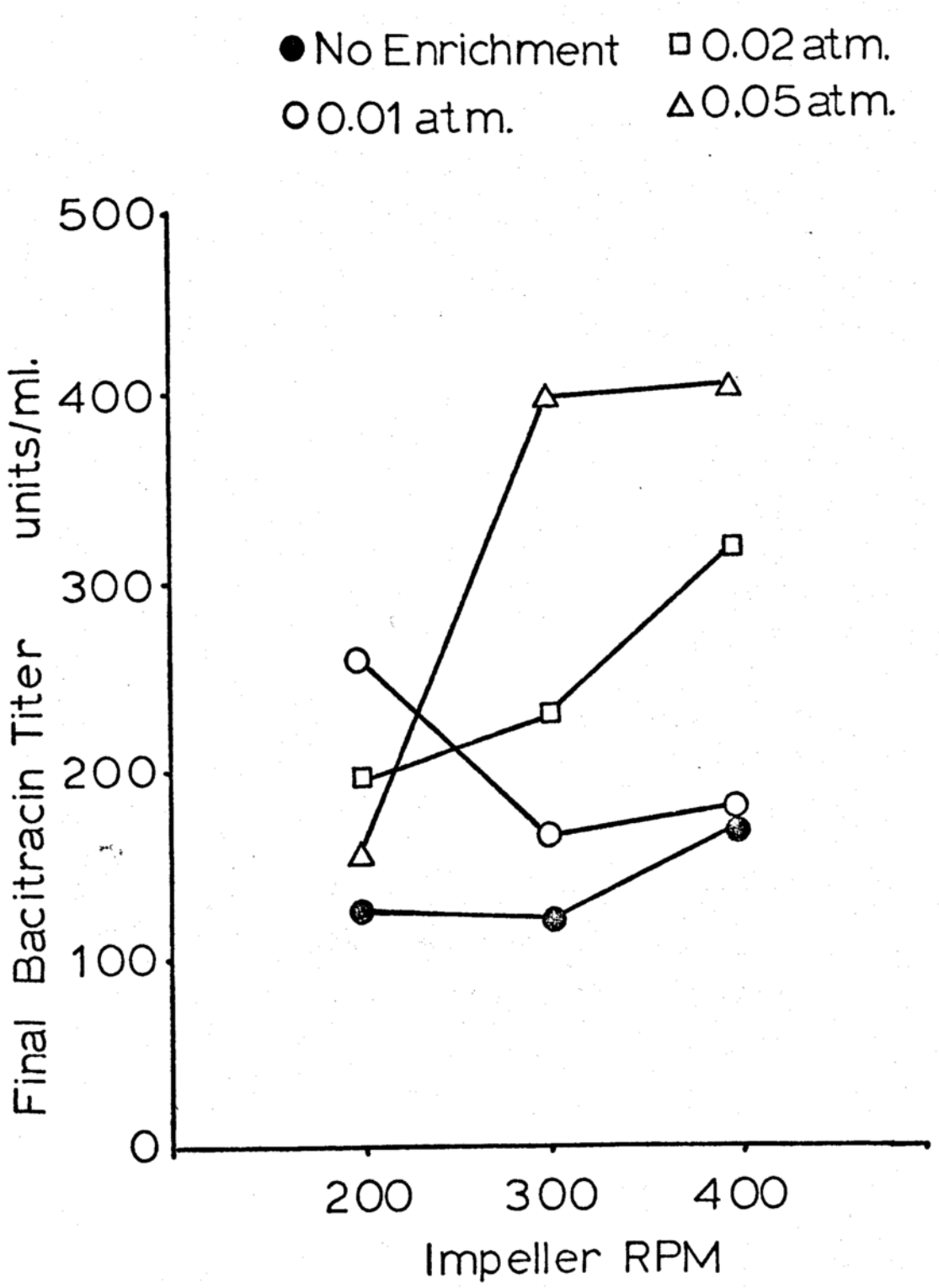
(1) Assume 50 units of bacitracin per mg.

(2) Grams of bacitracin gained per gram O_2 sparged through fermenter

(3) Grams of bacitracin gained per gram of enriched O_2 taken up

(4) Percent of enriched O_2 taken up by the culture

FIGURE 49


● No Enrichment
□ 0.02 atm.
○ 0.01 atm.
△ 0.05 atm.
Final Bacitracin Titer units/ml.
500
400
300
200
100
0
200
300
400
Impeller RPM

the power input was increased. (table XVII) The amount of enriched O_2 taken up by the culture remained between two and eighteen percent. Slightly more oxygen was taken up at the 0.01 atm. level than at either the 0.02 atm. or 0.05 atm. levels at lower rpm.

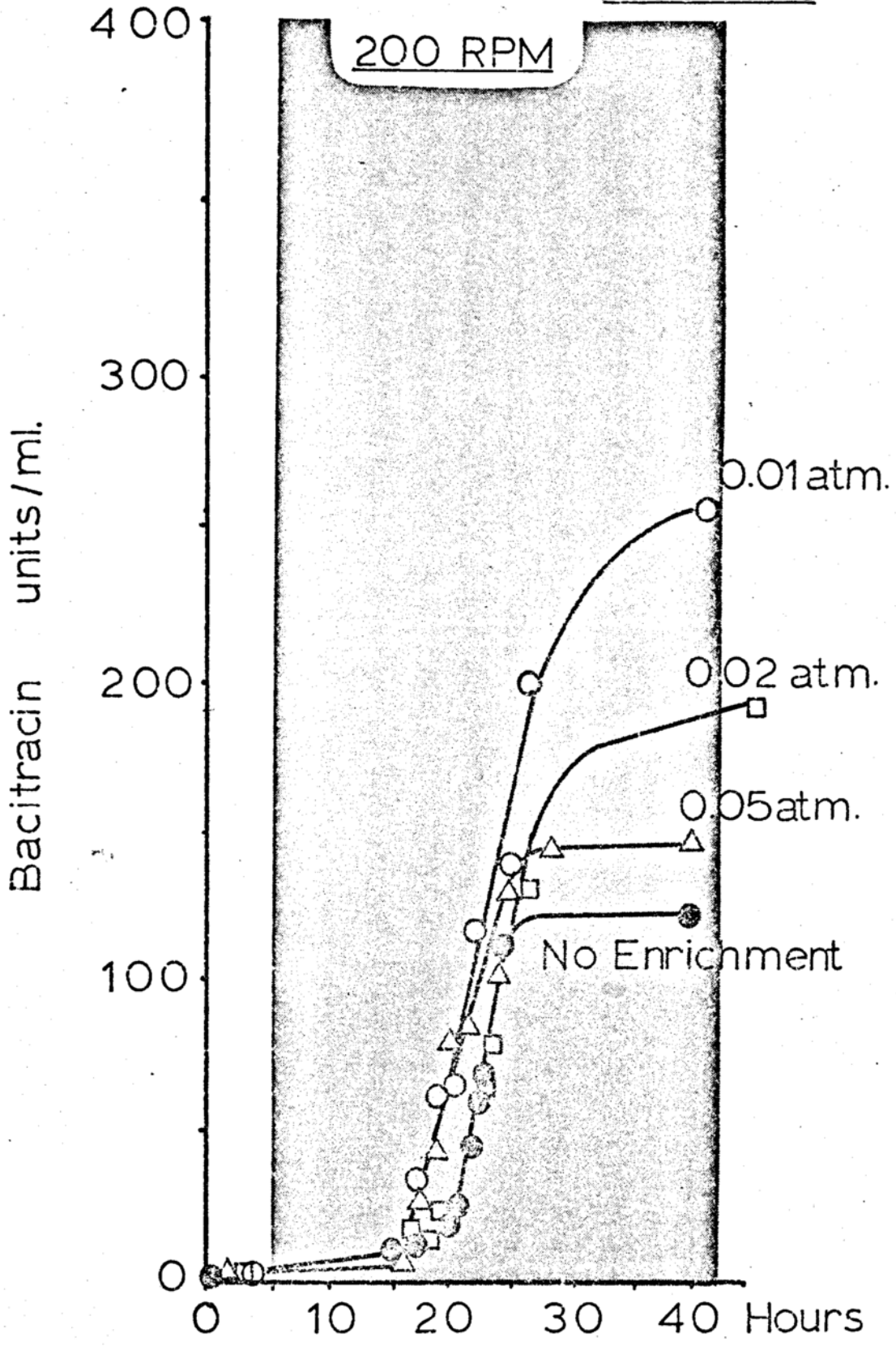
The maximum rate of antibiotic accumulation was affected by oxygen-enrichment. Figures 50, 51, and 52 summarize the effect of different levels of DOT control on the rate of bacitracin production. While O_2 enrichment did not increase the maximum rate at 200 rpm, at 300 rpm and 400 rpm the rate was increased two to four fold. Increasing levels of O_2 enrichment resulted in increased maximum rates of production. (Table XIX)

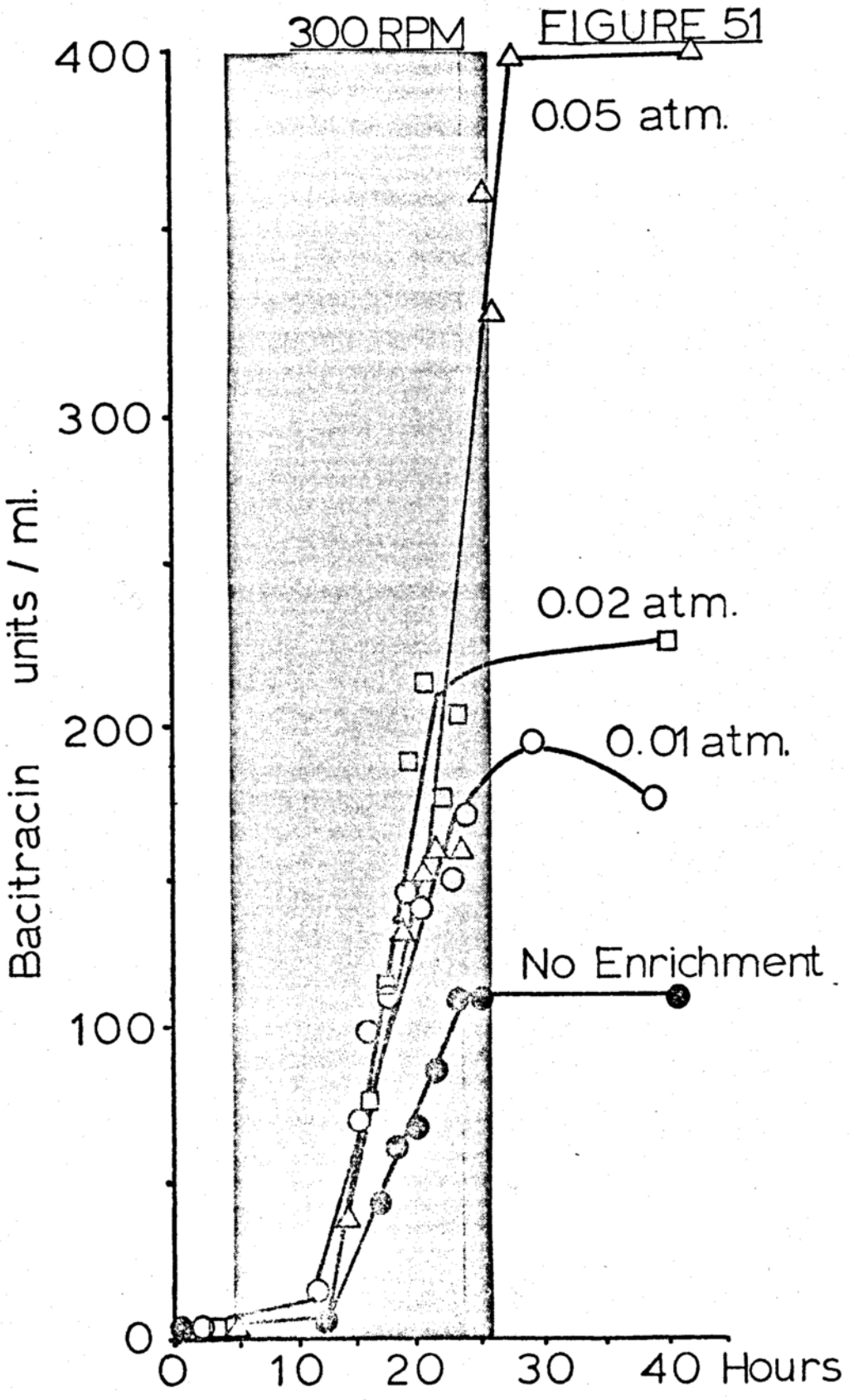
c. Utilization of Medium Carbohydrate

The disappearance of sucrose from the medium (assayed by the anthrone method, page 56) during the B. licheniformis fermentation at 200 rpm impeller speed suggested that medium carbohydrate may not be utilized during the first 15 hours. Paper chromatographic analysis of carbohydrates present in the medium during this fermentation suggested that: 1) during the first 15 hours only free glucose and sucrose was found in the medium, little fructose was detected, 2) after 15 hours of incubation, large quantities of a polysaccharide could be detected in the medium, no disaccharides were observed, and 3) traces of free glucose could be detected in several fermentations between 15 and 26 hours of incubation during the period when polysaccharide was present. No free fructose was observed during any part of the fermentation.

These results inply that B. licheniformis 10716 may utilize sucrose in a similar manner as reported for B. subtilis 168 (105) (figure 53). In this system sucrose is assimilated by two extracellular enzymes, a transfructosyl-levansucrase which cleaves the disaccharide liberating glucose which is transported into the cell. Fructose is not released

FIGURE 50


200 RPM
400
300
200
100
0
Bacitracin units/ml.
0.01 atm.
0.02 atm.
0.05 atm.
No Enrichment
0
10
20
30
40
Hours


300 RPM
FIGURE 51
0.05 atm.
0.02 atm.
0.01 atm.
No Enrichment
Bacitracin units / ml.
400
300
200
100
0
0
10
20
30
40 Hours

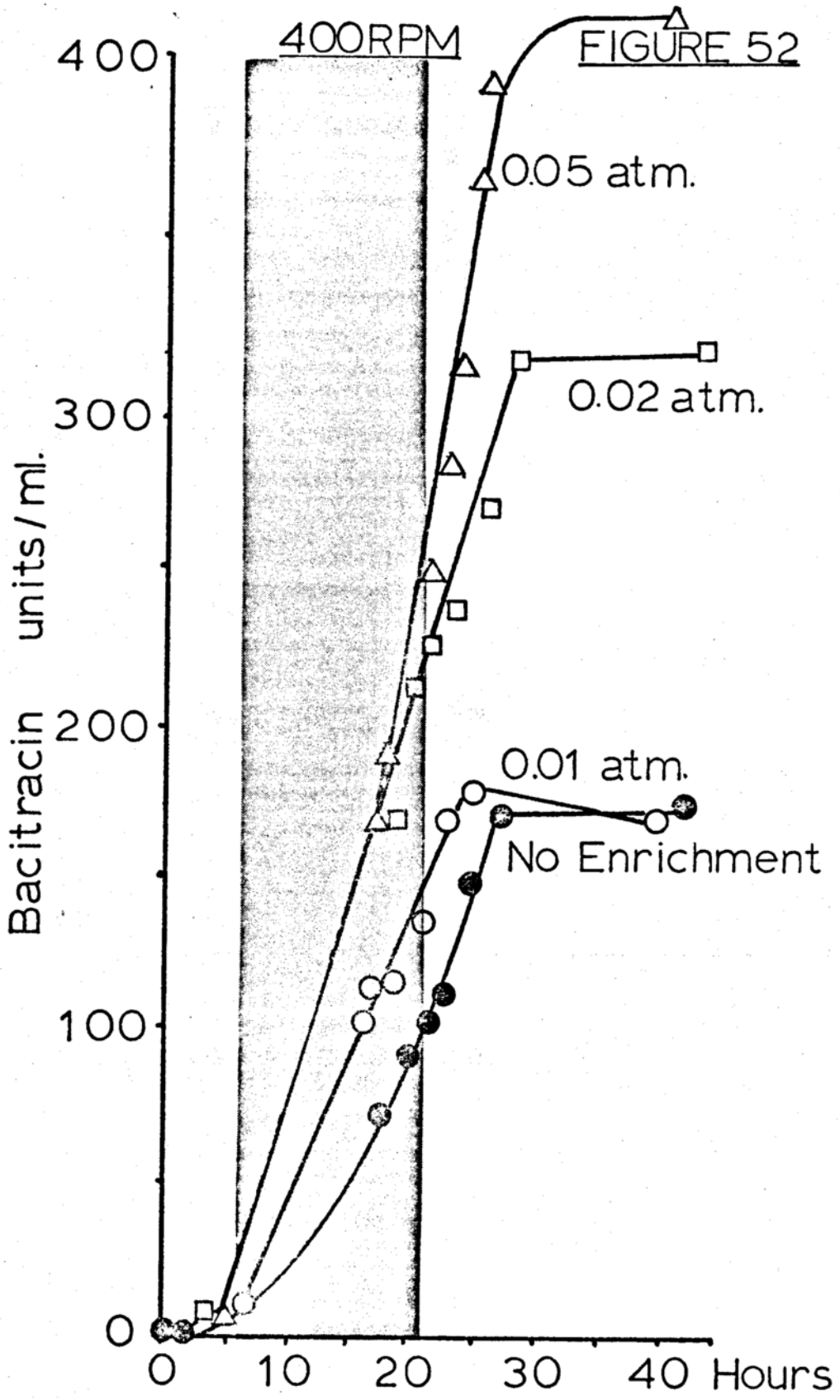
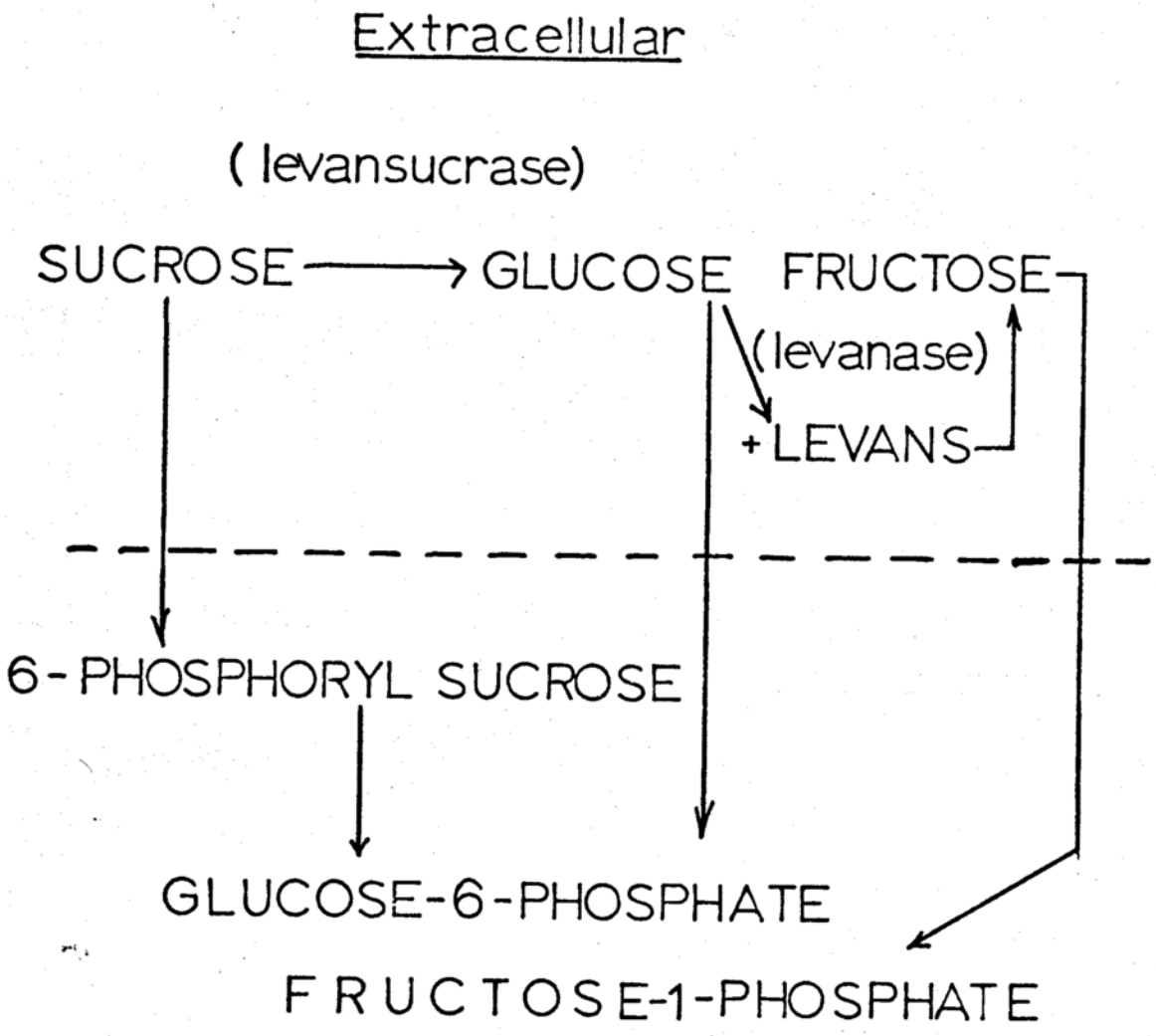

400RPM
FIGURE 52
0.05 atm.
0.02 atm.
0.01 atm.
No Enrichment
Bacitracin units/ml.
400
300
200
100
0
0
10
20
30
40 Hours

Table XIX

SUMMARY OF THE EFFECT OF OXYGEN-ENRICHED AERATION ON THE MAXIMUM RATE OF BACITRACIN PRODUCTION BY *B. licheniformis*

IMPELLER RPM	LEVEL OF ENRICHMENT	MAXIMUM RATE OF BACITRACIN ACCUMULATION UNITS/ml./HOUR
200	None	26
200	0.01 atm.	19
200	0.02	17
200	0.05	17
300	None	10
300	0.01	15
300	0.02	34
300	0.05	45
400	None	13
400	0.01	10
400	0.02	17
400	0.05	26

FIGURE 53


Extracellular
(levansucrase)
SUCROSE
GLUCOSE
FRUCTOSE
(levanase)
+ LEVANS
6-PHOSPHORYL SUCROSE
GLUCOSE-6-PHOSPHATE
FRUCTOSE-1-PHOSPHATE
Innercellular

from the enzyme into the medium but is transferred to another fructose residue forming levans of various lengths. After exhaustion of free glucose from the medium, an extracellular levanase cleaves the polysaccharide liberating fructose for utilization by the culture.

The operation of a sucrose system in B. licheniformis 10716 similar to that described for B. subtilis 168 would help to explain both the CO_2 evolution and sucrose utilization data observed for this fermentation (figure 54). Two peaks of CO_2 evolution are found. The first peak occurs during the first fifteen hours of incubation and could represent microbial respiration during the period of utilization of liberated glucose and extracellular levan synthesis. The reaction of the fructose polymer with the anthrone reagent results in a period of an apparent lack of carbohydrate utilization. (the first 15 hours of incubation) The decrease in anthrone reactive carbohydrate after 15 hours of incubation is accompanied by a second peak of CO_2 production. This second peak could represent respiration during a slow utilization of levan from the medium by this culture.

The effect of oxygen-enriched aeration on the utilization of sucrose is summarized in figures 54, 55, and 56. The level of residual carbohydrate in the medium after 40 hours of incubation (air sparge) could be reduced from 35 percent to 0 percent by increasing the impeller speed. At the higher impeller speeds very little lag in carbohydrate utilization was observed when sparging with air. A lag time of 10 to 15 hours was seen at 300 and 400 rpm when O_2 enrichment was used. At these higher impeller speeds, less carbohydrate was utilized by the culture when oxygen-enrichment was applied. The level of enrichment did not appear to significantly change the level of anthrone reacting carbohydrate remaining. At 200 rpm impeller speed, O_2 enriched aeration did not affect the rate or extent of carbohydrate utilized.

FIGURE 54

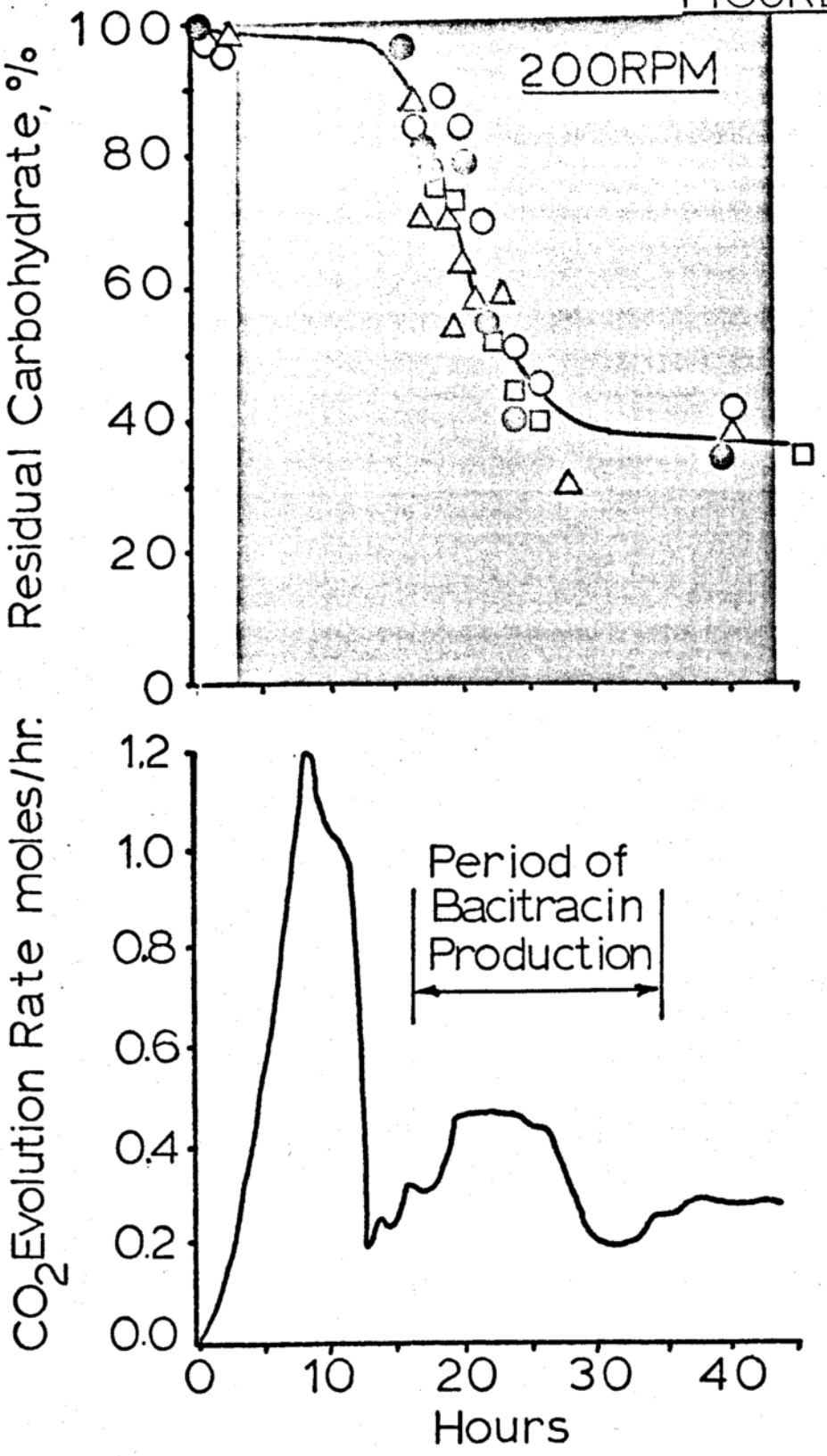

200RPM
Residual Carbohydrate, %
100
80
60
40
20
0
CO_2 Evolution Rate moles/hr.
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Period of Bacitracin Production
0
10
20
30
40
Hours

FIGURE 55

300 RPM

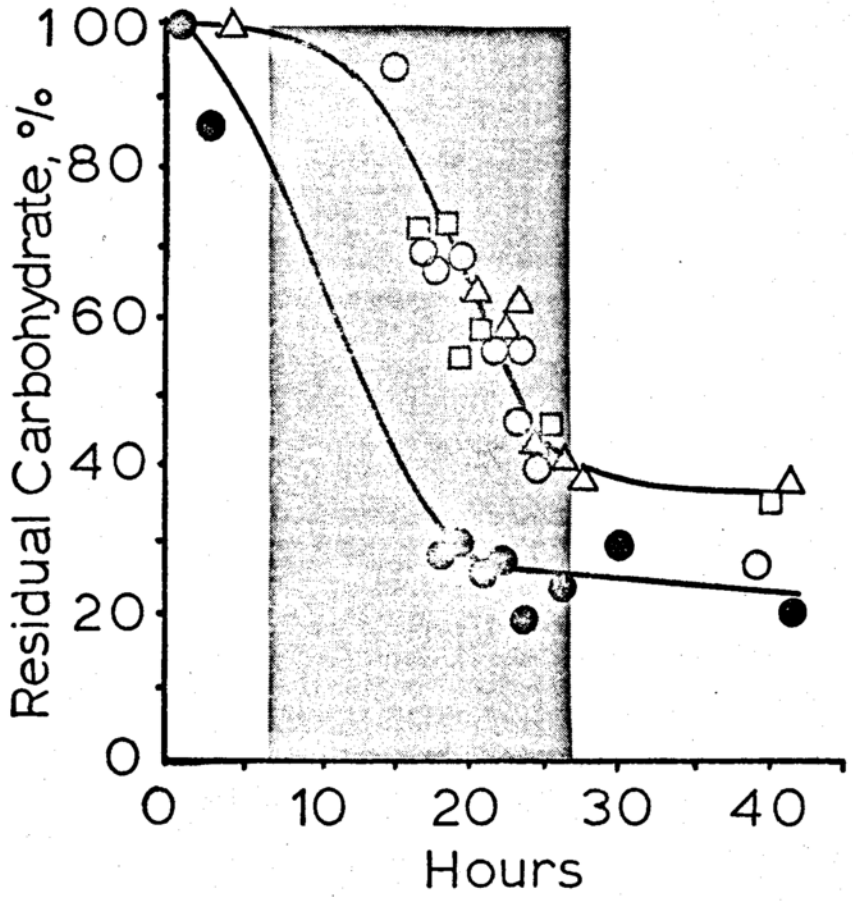
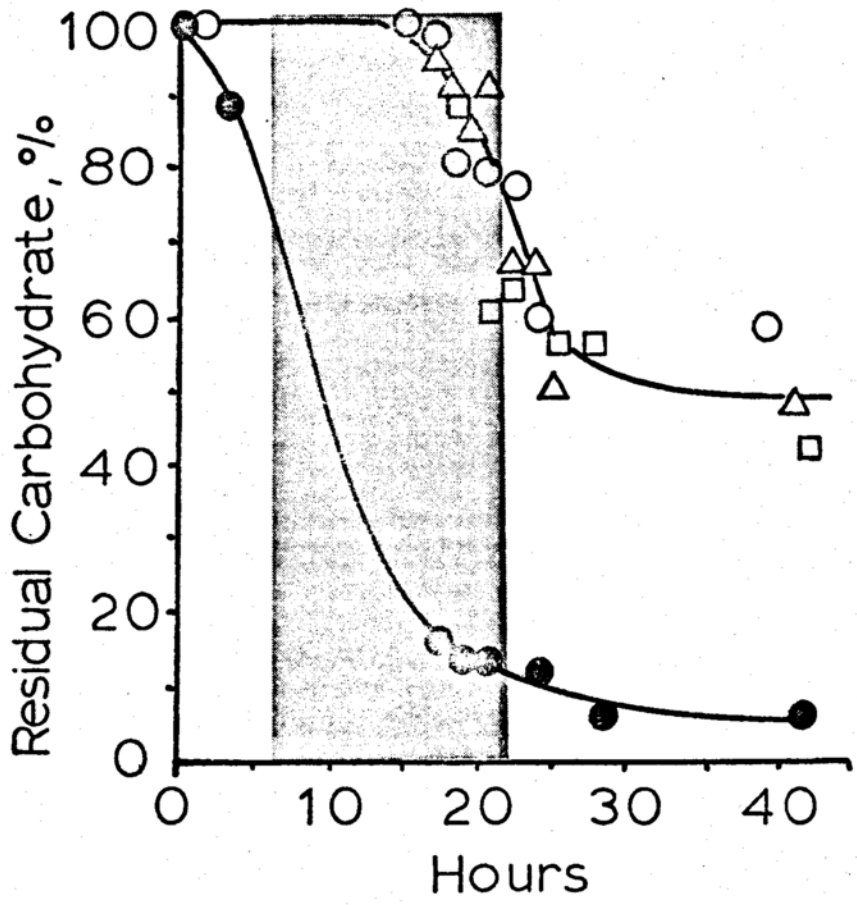


FIGURE 56

400 RPM



d. The Yield on Oxygen

Interpretation of the oxygen uptake rate data for the bacitracin fermentation was complicated apparently by the method of carbohydrate utilization of this culture. The OUR behavior was similar to the CO_2 evolution data in that two peaks of oxygen uptake occurred during this fermentation when sparging with air alone. These two regions of rapid oxygen uptake corresponded in time to the two periods of rapid CO_2 evolution. Between these two regions was a period of six to eight hours of very low oxygen uptake.

This pattern of oxygen uptake was not changed by application of oxygen-enriched aeration. However, because of this behavior, it was not possible to determine whether the automatic method of O_2 enrichment produced a period of suppressed OUR as was observed in some of the S. fradiae fermentations.

The OUR data in table XVII shows that only two to 18 percent of the added compressed O_2 was taken up by the culture. The grams of bacitracin gained per gram of compressed O_2 taken up is the yield of antibiotic on oxygen, ($Y_{bacitracin_{O_2}}$). Table XVIII summarizes the cell yield on oxygen and the antibiotic yield on oxygen for the entire 40 hour fermentation period. Both the cell yield ($Y_{cell_{O_2}}$) and the bacitracin yield on oxygen can be combined (106) to express the grams of antibiotic produced per gram of cell mass formed ($Y_{bac/cell}$).

At each of the three impeller speeds examined, the product yield per gram of cell mass formed increased as the level of O_2 enrichment was increased. (table XVIII) Examination of $Y_{bac/cell}$ suggests that the increase may be due to a decrease in the cell yield on O_2. The yield of bacitracin on oxygen remained relatively constant as the level of O_2 enrichment or the impeller speed was varied. However, the yield

Table XVIII

YIELD ON OXYGEN

RPM	ENRICHMENT	$Y_{CELL_{O_2}}$	$Y_{BACITRACIN_{O_2}}$	$Y_{BAC/CELL}$
200	None	0.16	0.14	0.89
200	0.01(atm.)	0.11	0.28	2.5
200	0.02	0.08	0.15	1.8
200	0.05	0.08	0.12	1.5
300	None	0.10	0.08	0.8
300	0.01	0.09	0.11	1.3
300	0.02	0.06	0.13	2.2
300	0.05	0.07	0.19	2.8
400	None	0.06	0.08	1.3
400	0.01	0.04	0.06	1.5
400	0.02	0.04	0.12	2.7
400	0.05	0.03	0.12	3.7

$Y = \frac{\text{grams of cells or bacitracin}}{\text{grams } O_2 \text{ consumed}}$

$Y_{bac/cell}$ = product yield per cell mass formed

of cells on oxygen decreased with increasing levels of DOT control using oxygen-enriched aeration.

These values for the cell yield on oxygen during the entire course of the fermentation are lower than normally reported for aerobic fermentations (0.75-1.0). When the yield on oxygen is calculated during the period of increasing cell mass only, a value for $Y_{cell_{O_2}}$ of approximately 0.6 is obtained for these fermentations.

The approximate material balance for oxygen for these fermentations can be assumed to be similar to that for the neomycin fermentations:

$$O_{2(\text{total oxygen uptake})} = O_{2(\text{cell mass})} + O_{2(\text{evolved } CO_2)}$$

The oxygen derived from the soy grits and sucrose can be ignored from this approximation as its oxidation level is approximately the same as that of the extracellular peptides produced. The quantity of oxygen evolved as dissolved CO_2 was not calculated because of the changing pH and because the CO_2 absorption coefficient for this medium was not determined (107).

The percentage of oxygen taken up by the culture recovered as either cell mass (assume 27 percent oxygen) or evolved CO_2 was calculated for both air and air + O_2 fermentations at all three impeller speeds. The average percentage of oxygen recovered was 79 percent with a range of from 62 to 95 percent.

e. Production of Lactic Acid

The production of lactic acid by B. licheniformis was studied in order to investigate whether O_2 enrichment significantly decreased the production of more reduced products during the course of the fermentation.

Using air alone, increasing impeller speed reduced the maximum lactic acid produced from 2.4 grams per liter at 200 rpm to 1.8 grams per liter at 400 rpm. The production of lactic acid by B. licheniformis

peaked after 25 hours of incubation and decreased slowly during the remaining 15 hours. O_2 enrichment at a level of 0.01 atm. had no effect on reducing the amount of lactic acid produced at any impeller speed examined. Oxygen-enrichment at a level of 0.02 atm. and 0.05 atm. accelerated the decrease in lactic acid levels during the last 15 hours only at 200 rpm. At this low impeller speed O_2 enrichment was still continuing after 25 hours. At the two higher impeller speeds, O_2 enrichment had ceased before peak lactic acid accumulation occurred. The rate of lactic acid accumulation also was not changed during the period of oxygen enrichment. This lack of effect was unusual as the pH behavior of the air and air + O_2 fermentations were significantly different (sections 3a). The production of acetic acid or pyruvic acid from sucrose (99) was not monitored.

f. The Effect of Oxygen-Enrichment on the Ratio of Bacitracin Produced

Neutral n-butanol extracts from fermentation harvest samples were examined by thin layer chromatography for the effect of oxygen-enriched aeration on the products formed. The five to eight ninhydrin positive spots separated were examined for biological activity by bioautography (page 38). The ratio of bacitracin A, B, and F was determined by high pressure liquid chromatography (page 49).

Bioautography of TLC samples demonstrated only two microbiologically active compounds in all bacitracin fermentations. These two compounds were identified by R_f with standard bacitracins as being bacitracin A and B or A+B and some C. The O_2 enriched fermentations did not form any additional microbiologically active products.

Table XX summarizes the effect of oxygen-enrichment on the ratio of bacitracin A and B produced by B. licheniformis 10716. O_2 enrichment only appears to be able to affect the ratio of products produced under

Table XX

THE EFFECT OF OXYGEN-ENRICHED AERATION ON THE RATIO OF BACITRACIN A, B, AND F PRODUCED BY *Bacillus licheniformis* 10716

IMPELLER RPM	LEVEL OF O_2 ENRICHMENT	PERCENT BACITRACIN: A[1]	B[1]	F's[2]	FINAL pH
200	None	66	34	60	7.1
200	0.01 atm.	45	55	57	7.2
200	0.02	69	31	34	7.2
200	0.05	71	29	69	7.8
300	None	70	30	62	8.4
300	0.01	71	29	66	8.7
300	0.02	70	30	62	8.8
300	0.05	69	31	60	8.8
400	None	72	28	50	8.8
400	0.01	70	30	58	8.9
400	0.02	72	28	49	8.8
400	0.05	62	38	40	8.8

(1) Percent of microbiologically active bacitracins

(2) Percent of total bacitracins separated on HPLC of fermentation extract

conditions of poor oxygen transfer (200 rpm impeller speed). At 300 rpm and 400 rpm, enrichment of the aeration did not change the approximately 70 percent A and 30 percent B ratio of bacitracins produced. The percentage of bacitracin F present in all bacitracin fermentations was not significantly changed by oxygen-enriched aeration even though the conversion of bacitracin A or B to the F form has been suggested to involve molecular oxygen (95). The high percentage of the F forms (34 to 70 percent) may be due to the high final pH of these fermentations. Commercial bacitracin fermentations controlled at pH less than 7.5 may contain 5 to 20 percent F (43).

Since the air and air + O_2 fermentations differed in pH behavior, onset of sporulation, and carbohydrate utilization, the data in table XX suggests that none of these factors affects the ratio of bacitracin A to B produced by B. licheniformis.

VI. DISCUSSION

A. The Effect of Oxygen-Enriched Aeration on Increased Process Productivity

The data in figures 18, 28, and 49 demonstrate that increasing the driving force of oxygen transfer by enrichment of the aeration with compressed oxygen can increase the productivity of each of the three fermentations studied. The molar conversion of glycerol to DHA could be increased from 33 percent to 100 percent, the yield of neomycin by 25 percent, and the yield of bacitracin by 235 percent. These increases were obtained by maintaining the dissolved oxygen tension at a constant level with O_2 enriched air without increasing agitation speed or total aeration rate. Both an increase in final product yield and an increase in production rate were observed for the production of neomycin and bacitracin. The rate of neomycin biosynthesis could be increased by a factor of two and bacitracin production by a factor of almost four.

The data from tables VIII, IX, XI, XVII, and XVIII indicate that the increase in the amount of product formed was due to an increase in the specific productivity of each culture when exposed to an increased driving force of oxygen transfer. More product was produced with the production of less cell mass. The specific conversion of glycerol to DHA increased from 12.2 grams DHA per gram of cell mass at the point of maximum conversion to 35.8 with oxygen-enrichment. The Y (antibiotic/cell mass) for both antibiotic fermentations increased from 0.18 to 0.28 for neomycin production and from 1.3 to 3.6 for bacitracin production.

The reason why these effects are observed is not clear. Oxygen-enrichment of the aeration was made in response to the oxygen demand of the culture, $\frac{\mu X}{Y_{o_2}}$; in other words, cellular respiration. However, for the three

processes studied, the relationship between respiration and product formation is unknown. The results for the production of neomycin and bacitracin indicate that the greater availability of oxygen during the period of peak respiratory activity of these cultures somehow affects the biochemical activity of these organisms later during the fermentation.

The concept that for non-growth associated products the availability of oxygen during the early period of growth influences the productivity of the culture later in the fermentation was studied by Rolinson (109). This early work suggested that the availability of dissolved oxygen during the period of growth of Penicillium chrysogenum influenced both the quantity and the kind of respiratory enzymes synthesized. These enzymes apparently influenced the rate of penicillin production and respiratory activity later during the fermentation. Mycelium grown under conditions of low aeration and subsequently subjected to an increased supply of available oxygen continued to respire at a slower rate than mycelium grown initially under conditions of higher aeration. Respiration was apparently limited by the magnitude of the existing respiratory mechanism which appeared to be synthesized in proportion to the concentration of available oxygen during the growth of the organism.

Increased specific productivity in response to increased pO_2 would imply that the microorganisms were somehow responding to the partial pressure of oxygen in the sparge gas not only to the level of dissolved oxygen. Tables VIII and IX suggest that increased pO_2 can affect enzyme activity (glycerol dehydrogenase) independent of cell mass. Increasing the period of oxygen limitation by sparging with decreased oxygen partial pressures resulted in decreased conversion of glycerol to DHA but the production of more cell mass. This may indicate that the pO_2 of the aeration can function, by direct enzyme regulation, in directing the path of glycerol in metabolism either to accumulation of DHA or to the production of cell mass.

DHA or to the production of cell mass. The data also indicate that the conversion of glycerol to DHA can be manipulated without affecting other aspects of cell metabolism--the rate of the accumulation of acuduc products.

Increased levels of pO_2 have previously been shown to be capable of enzyme regulation in the induction of pigment production in Klebsiella aerogenes (110) and the induction of cytochromes in yeast (111). Recently Tsao (112,113,114,115,116) and Minura (117) have demonstrated that the direct absorption of oxygen by microorganisms concentrated in the static film layer surrounding a gas bubble can be an important factor in overall oxygen transfer. Their studies have demonstrated that during exponential growth 90 percent of the oxygen taken up by the culture may be by direct absorption. If oxygen transfer by direct absorption can occur, then it may be possible for the microorganisms to be biochemically regulated by the pO_2 of the sparge gas.

This effect of increased partial pressure of oxygen may further point to the ability to direct the course of some microbial processes toward accumulation of more desired metabolites with the elimination of undesirable products independent of the production of cell mass. The data on the ratio of antibiotics produced under conditions of O_2 enrichment (tables XIV and XIX) suggest that the pathway of carbohydrate derived antibiotics (neomycins) may be able to be manipulated by pO_2. However, neither the thin layer chromatographic method nor the HPLC method completely separated the bacitracins. The separation of these peptide antibiotics was not as good as the GLC separation of neomycin B and C. Since the actual structures of all of the bacitracin peptides produced by B. licheniformis have not been determined, it is difficult to conclude from the data whether or not increased pO_2 affects the ratio of the production of these amino acid derived products. The data does suggest that for both antibiotic fermentations studied, when the fermentation becomes oxygen limited, the ratio of

limited, the ratio of antibiotics produced changes.

B. The Effect of Oxygen-Enriched Aeration on Microbial Physiology

The nature of the various interactions between oxygen and microbial cells are still largely unknown. Molecular oxygen has been observed to act as an electron acceptor, a nutrient, an enzyme regulator (131), and as an inhibitor of microbial growth (110,128-133).

The CO_2 evolution data from this study (figures 23 - 26, and 41 - 44) and the oxygen uptake rate data (figures 32 - 35) suggest that O_2 enrichment strongly affects microbial respiration. Increasing the driving force of oxygen transfer increased the rates of oxygen uptake, carbon dioxide evolution, with a slightly depressed respiratory quotient.

Increased oxygen uptake rates while using pure oxygen for aeration have previously been reported by Shu (6) during the production of ustilagic acid, α-amylase, and citric acid. The observed OUR for these fermentations ranged from 1 to 3.3 fold greater in the presence of oxygen sparging than with air. Similar increases were observed for the O_2 enriched G. melanogenus fermentations and for both antibiotic fermentations during the period of enrichment. This is not surprising as the oxygen uptake rate depends not only on the biological oxygen demand but also on the rate of oxygen supply. The former depends on the cell concentration as well as the specific oxygen demand of the organism-the maximum rate of oxygen utilized per gram of cell dry weight. The rate of oxygen supply can be increased by manipulation of environmental factors, in this case the pO_2 of the sparge gas.

The amount of enriched O_2 actually taken up by the culture (figures 32-35, table XIII) appeared to depend on how the compressed oxygen was added. Too much oxygen-enrichment too early in the fermentation appeared

appeared to suppress O_2 uptake while a small increase in the pO_2 of the sparge gas did not.

Increased O_2 evolution when sparging with gas mixtures with partial pressures of oxygen greater than air has also been reported in the literature (118). Accumulation of evolved CO_2 in the fermenter head space and the culture medium can inhibit many microbial biosynthetic processes (118,119,120,121,122). Problems associated with inadequate ventilation of evolved CO_2 are now being studied for industrial processes (123,124,125,126). The data in figure 41 suggests that there is a direct relationship between the increased driving force of oxygen transfer and the amount of additional CO_2 evolved during the period of enrichment. For the B. licheniformis fermentation at 200 rpm and 300 rpm impeller speed, as the average driving force of oxygen transfer was doubled, the culture increased CO_2 evolution 2.6 fold. However, there appears to be an upper limit to this effect in this system. At 400 rpm impeller speed, doubling the driving force of O_2 transfer only increased the CO_2 evolution rate by a factor of 1.25. With the slower growing Streptomyces fradiae, the relative CO_2 evolution rate during the period of O_2 enrichment was increased only at low agitation speeds (100 to 200 rpm) where O_2 transfer was poor. Under better conditions of oxygen transfer, O_2 enrichment did not significantly affect CO_2 evolution during the period of enrichment.

There is no indication from the CO_2 evolution data from either the neomycin or bacitracin fermentation that levels of carbon dioxide inhibitory to antibiotic biosynthesis were produced in response to O_2 enrichment under the conditions studied.

Examinations of figures 23-26, and 41-44 reveals that oxygen- enriched aeration not only increased the peak level of CO_2 produced but the culture evolved CO_2 at a level of two to five fold greater than during the corresponding period in the air fermentation. Of particular

the CO_2 evolution rate of the O_2 enriched fermentations after enrichment has ceased. The data in figures 24, 25, 43 and 44 suggest that the CO_2 evolution rate of the O_2 enriched fermentations drops significantly below that of the air fermentations when enrichment ceases. This indicates that the respiratory mechanism of these cultures is changed in response to the increased pO_2 of the sparge gas.

Increased partial pressures of oxygen also appear to have a definite effect on the rate of microbial growth. The specific growth rate of G. melanogenus decreased abruptly at the onset of enrichment from 0.23 $hour^{-1}$ to 0.06 $hour^{-1}$. The maximum growth rate of B. licheniformis was dramatically decreased by exposure to elevated pO_2 levels. (figure 40). This inhibitory effect appears to be greatest under better oxygen transfer conditions (higher impeller speed) decreasing the growth rate of the Bacillus from 0.65 $hour^{-1}$ to 0.14 $hour^{-1}$. Insufficient cell dry weight data was obtained from the S. fradiae fermentations to determine the effect of O_2 enrichment on the growth rate of this mycelial fermentation. The final cell dry weight yield, however, was not decreased by enrichment.

The mechanisms for oxygen toxicity are not well understood probably because they are very diverse. Oxygen toxicity for aerobic organisms has been attributed to autoxidation of cytochromes, oxidation of thiol groups, enzyme inactivation (127), lipid peroxidation and free radical accumulation (108). In addition, oxygen toxicity symptoms have also been attributed to over oxidation of the NAD^+/NADH couple (128). The inhibitory effect of hyperbaric oxygen on the growth of microorganisms has been documented in the literature (129,130,131,132,133,134). The majority of work concerns organisms grown on agar plates with little information concerning the effect of elevated pO_2 on the growth rate of microorganisms in submerged culture.

In organisms such as Gluconobacter species which lack either a normal

normal glycolytic path or a tricarboxylic acid cycle, the known energy yielding phosphate esterifying reactions are few in number. The primary phosphorylations appear to be at the substrate level (79,80) Hauge (79,80) has demonstrated the lack of a requirement for NAD with glycerol dehydrogenase. He also presented evidence of suggest that this oxidation may be coupled directly to a dytochrome with intervention of a flavoprotein presumably necessary for the latter reaction. If G. melanogenus relies primarily on substrate level phosphorylations as its source of phosphate esters, then oxygen sensitivity of its cytochromes could have immediate toxic effects on cell growth.

Little information exists in the literature on the effect of oxygen on sporulation of Bacilli. Whether the earlier onset of sporulation of B. licheniformis is the result of some toxic effect of O_2 enrichment for this organism cannot be determined from this data. This change in sporulation may reflect the effect of O_2 enrichment on the growth rate of the culture.

There is also no evidence for either antibiotic fermentation that oxygen-enrichment can significantly increase the rate of nutrient utilization by the producing organisms. This situation may be different if monosaccharides were employed instead of polysaccharides requiring extracellular enzyme systems for assimilation.

C. Future Prospects

The application of oyxgen-enriched aeration could result in significant advantages for other microbial processes. In addition to increasing the productivity of existing fermentation equipment, savings of power input could be realized. The oxygen sensitivity of each culture would have to be determined as well as the method of addition of oxygen in order to maximize the efficient of utilization of enriched O_2 without inhibition of cell growth or repiration. Of

cell growth or respiration. Of greatest interest is the unexplored potential for the use of oxygen-enriched aeration to regulate microbial metabolism and product formation. Further investigation and application of this technique could lead to significant changes in the design of industrial fermentations.

Part II. BIOLOGICAL CONTAINMENT OF A CONTINUOUS CENTRIFUGE

I. BIOLOGICAL BARRIERS FOR PROTECTION OF PERSONNEL

A. Biohazards in the Production of Pharmaceutically Active Substances from Microorganisms

The production of pharmaceuticals and biologicals from known pathogenic or potentially pathogenic microorganisms must be undertaken with constant regard for worker safety. The offices of the Public Health Service and the USDA which have regulatory responsibility for quarantine and interstate shipment of etiologic agents have combined to supervise the handling of these agents. What has resulted is the "Classification of Etiologic Agents on the Basis of Hazard" (135). This publication not only classifies bacterial, fungal, parasitic, and viral agents on the basis of potential biohazard but also outlines the level of competence and physical containment recommended for each class (136,137).

The basis for classification is the following:

Class I: Agents of no or minimal hazard under ordinary conditions of handling

Class II: Agents of ordinary potential hazard, may produce disease from accidental inoculation or injection but which are contained by ordinary laboratory technique

Class III: Agents involving special hazard or agents derived from outside the U.S. which require federal permit for importation. This class includes pathogens which require special conditions for containment.

Class IV: Agents that require the most stringent conditions for their containment or may cause serious epidemic disease

Class V: Foreign animal pathogens excluded from the U.S. by law.

Table XXI lists some of the commercially available biologicals, their source organism and how each organism is classified by the government.

It can be seen that many of the organisms used in production of biologics are listed as in class III. This dictates that handling of these pathogens be carried out utilizing special conditions for containment. While the USPHS requires only ordinary laboratory techniques to control class II agents, class III agents are subject to the following more detailed conditions (135,136):

1. A controlled access facility, separation of agents from the general traffic pattern of the rest of the building or laboratory
2. Negative air pressure is maintained at the site of work in a preparation cubicle or under a hood. Air is recirculated only after it has been adequately decontaminated through high efficiency filters.
3. Animal experiments, including cage sterilization, refuse handling, disposal of animals, etc. are conducted with a level of precaution equivalent to conditions required for laboratory experiments.
4. Personnel at risk are immunized against agents for which immune prophylaxis is available.

The construction of equipment to contain etiologic agents of high biohazard has brought together scientists, engineers and military personnel producing a substantial volume of literature on all aspects of containment and control. The basic concepts to this work are the various types of biological safety barriers. A microbial barrier as described here is a device or system that will prevent or limit the passage of microbial

Table XXI COMMERCIAL BIOLOGICALS

Biologic	Source Organism(142)	Method of Culture	Government Classification
Typhoid Vaccine	Salmonella typhosa	liquid	II
Cholera Vaccine	Vibrio comma	liquid	II
Plague Vaccine	Yersenia pestis	liquid	III
Pertussis Vaccine	Bordetella pertussis	liquid	II
BCG Vaccine	Mycobacterium tuberculosis	liquid	III
Brucella Vaccine	B. abortus, B. meditensis, B. suis	liquid	III
Tuberculin	M. tuberculosis	liquid	III
Histoplasmin	Histoplasma capsulatum	liquid	III
Blastomycin	Blastomyces dermitides	liquid	II
Coccidiodin	Coccidioides immitis	liquid	III
Botulinum toxin	Clostridium botulinum	liquid	II
Diphtheria toxin and toxoid	Corynebacterium diphtheriae	liquid	II
Tetnus toxin and and toxoid	Cl. tetani	liquid	II
Trichinella extract	Trichinella spiralis	inoculated rodents	II
Small Pox vaccine	vaccinia virus	chick embryo	II
Yellow Fever vaccine	yellow fever virus	domestic foul embryo	II,III, IV
Rabies vaccine	rabies virus	rabbit brain, duck embryo	II,III
Influenza vaccine	influences viruses	chick embryo	II
Poliomyelitis vaccine	poliomyelitis virus	primary monkey kidney	II
Measles vaccine	Rubeola or Rubella virus	avian embryotic tissue	II
Mumps vaccine	mumps virus	chick embryo	II
Typhus vaccine	Rickettsia prowazeki	domestic foul yolk sac	III

contaminants. These barriers are either primary barriers that immediately surround the hazardous procedure or secondary barriers that surround the primary barrier and provide additional assurance of the containment or exclusion of microbial elements.

The most important primary barrier available to the microbiologist for containment is the ventilated cabinet or hood. Secondary barriers which include laboratory isolation, room design, ventilation, contaminated waste treatment, animal housing and disposal, and specifics of mechanical services designed for containment of biohazardous material are thoroughly discussed by Runkle and Phillips (138). Laboratory design is also discussed by Chatigny (139), Hellerman (140), and Wedum (141).

B. The Safety Hood as a Primary Biological Barrier

The hood enclosure is the single most important device for worker safety in the microbiological laboratory. Safety hoods may range from the open-front "fume hood" enclosure to tightly sealed, interconnected hood systems often referred to as cabinets rather than hoods. Workers in this field have defined three levels of control systems (138,139,143):

Class 1: Open-front, ventilated hoods including vertical laminar flow hoods

Class 2: Open-front cabinets with restricted entry area and with provision for attachment of gloves. Some uncontrolled leaks are permitted, but the direction of leakage flow is controlled.

Class 3: Closed cabinet systems in which all leaks in and out are through controlled openings. Gloves are permanent fixtures and entry and exit are through air locks, autoclaves or disinfectant bath.

Many common bacteriological and viral laboratory techniques produce aerosols. Operations such as pipetting, operation of the Waring Blendor, intranasal instillation of culture suspensions into mice, egg inoculation, egg harvest, serial diluting, centrifugation, shake flask culture, lyophylization, and grinding of tissue produce undeniable aerosols. Liberation of these aerosols results in heavy contamination of the workers hands, work surfaces, and of animals, eggs or culture vessels. Wedum (141) has collected and tabulated much of the literature on aerosol generation from simulated trial manipulations. Quantitatively, the results showed wide variations in number of colonies observed when the surrounding air was sampled using slit samplers (144).

Selection of a hood system requires considerable care. For many microbiological operations (considering safety alone) the open-front class 1 unit is adequate but only if designed, installed, and operated properly (139). If aerosols are being generated at elevated pressures or with strong sprays class 2 or class 3 measures may be desirable. The entirely closed cabinets (class 3) offer a great deal of improved protection and are less subject to accidental misuse. Their major deficiencies are very high cost, restricted work space, difficulty of passing materials in and out without breaking the protective barrier, and worker reluctance to use the equipment properly. No level of safety design can allow for effective containment in the absence of rigorous microbiological technique by the operator.

With the safety of personnel and above factors in mind, Wedum (141) has recommended various hoods be used depending on the agent being manipulated. His suggestions are reproduced in table XXII.

Among the factors most critical for the design of the safety enclosure is air flow. For class 1 and 2 cabinets air velocity and volume are key factors. Air velocity into the hood must overcome or

Table XXII

CORRELATION OF ESTIMATION OF RISK WITH RECOMMENDATIONS FOR USE OF PROTECTIVE CABINETS (141)

Disease or agent	Cabinet system[1]	Single cabinets[2]	
	Aerosol studies	Aerosol studies	Other techniques
Brucellosis	+	-	+
Coccidioidomycosis	+	-	+
Russian spring-summer encephalitis	+	-	+
Tuberculosis	+	-	+
Monkey B virus	+	-	+
Glanders	+	+	+
Melioidosis	+	+	+
Rift Valley fever	+	+	+
Arbo viruses, general	-	+	+
Encephalitides, various	-	+	+
Psittacosis	+	+	+
Rocky Mountain spotted fever	+	+	+
Q fever	+	+	+
Typhus	+	+	+
Tularemia	+	+	+
Tularemia	-	+	+
Venezuelan encephalitis	-	+	+
Anthrax	+	-	+-
Botulism	+	+	+-
Histoplasmosis	-	+	+-
Leptospirosis	-	+	+-
Plague	+	-	+-
Poliomyelitis	+	-	+-
Rabies	+	-	+-
Smallpox	+	-	+-
Typhoid	-	+	-
Adeno and entero viruses	-	+	+-
Diptheria	-	+	-
Fungi, various	-	+	-
Influenza	-	+	+-
Meningococcus	-	+	-
Pneumococcus	-	+	-
Streptococcus	-	+	-
Tetanus	-	+	-
Vaccinia	-	+	-
Yellow fever	-	+	-
Salmonellosis	-	+	+-
Shigellosis	-	+	+-
Infectious hepatitis	-	-	+-
Newcastle virus	-	+	-

[1] 'Cabinet System' refers to a Class 3 enclosure
[2] 'Single Cabinet' refers to either a Class 1 or 2 open-front safety hood

factors. Air velocity into the hood must overcome or control diffusion of contaminants by local air currents from room ventilating systems or from operator activities in the immediate area. Each cabinet should have a fan and filter unit exclusively for its own volume. Class 3 systems should provide a minimum of ten volume changes per hour. These cabinets are generally run with a negative air pressure with respect to the room pressure of from 0.75 to 2 inches of water.

Treatment of exhaust air from contaminated control cabinets can be accomplished by filtration and/or incineration. Commercial filters known as ultra high efficiency particle arrestors (HEPA) have been microbiologically evaluated for penetration of submicron T bacteriophage. The percentage penetration varies from 0.002 percent to 0.00002 percent. Such filters therefore provide excellent protection against viral and microbial aerosols. Chemical testing of HEPA filters is accomplished using a dioctylphtalate fog (DOP test) of 0.3 micron particle size (154).

In specialized chambers where intentional aerosols of highly pathogenic organisms are artificially created it may be desirable to employ incineration of exhaust air (145,146,147). Since in the event of power failure or heater burn out the incinerator could fail and therefore could pass microorganisms without adequate treatment, an in-line filter is usually employed as a fail-safe device.

Table XXIII summarizes some recommended agents and treatments for sterilization or decontamination in microbial barriers. (138,148,149) Heat is the most effective and reliable method of inactivating microorganisms and should be used whenever possible. Ethylene oxide (ETO) can be employed in closed systems with excellent results. However, the gas is slow in killing microbes and usually must be mixed with other gases to avoid explosion hazards. Enclosures must be aerated for 24 hours or more before use after

Table XXIII

SOME RECOMMENDED AGENTS AND TREATMENTS FOR STERILIZATION OR DECONTAMINATION IN MICROBIOLOGICAL BARRIERS

Agent	Recommended treatments	Limitations of Use
Moist heat (autoclave, high vacuum)	135° C, 3-5 minutes	effective if material is pervious to steam
Moist heat (autoclave, no vacuum)	121°C, 15-30 minutes	not including come-up time or size of vessel
Dry Heat	160°C, 2 hours 170°C, 1 hour	other combinations of temperature and time are acceptable
Ethylene oxide gas (in a non-explosive gas mixture	25°-55°C, 300 mg/l 6-16 hours 30-60% R.H.	will not penetrate solids, adsorbed in rubber, plastic necessitating aeration if material is to contact skin
Peracetic acid spray	25°C, 2% with 0.1% surfacant, continuous for 20 minutes	corrodes many metals degrades to active acid, oxygen, and water
Steam-formaldehyde	25°C, 1 ml/cubic ft. R.H. above 80%, 30 minutes to 10 hours	formaldehyde polymerizes on surfaces often necessitating long aeration periods
Beta-propiolactone vapor	25°C, 200 mg/cubic ft. in air R.H. above 70%, 30 minutes to 2 hours	aeration required prior to entrance into area
Dunk-tank formalin (37% HCHO)	25°C, 10%, 10 minutes	irritating fumes
Sodium hypochlorite solutions	25°C, 500-5000 ppm with 1% surfacant, 5 minutes	corrodes many metals
Strong tincture of iodine	25°C, 10 minutes	stains many materials
Ultraviolet radiation	25°C, 800 microwatt per square cm.	low penetrating power limited to exsed surfaces and air, bulbs must be clean and checked

treatment with ETO to avoid irritating action of absorbed ethylene oxide on human tissue.

Also listed in table XXIII are agents employed for disinfectant dunk tanks. Each agent should be evaluated for a specific cabinet on the basis of reactivity with the materials of construction and effectiveness as a biological barrier with respect to the organism being manipulated in the enclosure.

Ultraviolet irradiation from low-pressure mercury tubes is useful in elimination of both surface and airborne contamination. U.V. lamps can be used successfully provided proper attention is given to the following factors:

1. The U.V. rays have poor penetration power. A layer of dirt or dust on the tube itself will reduce the output appreciably.
2. Air temperature and velocity affect both the U.V. output and the action on airborne microbes (poor activity at high humidity).
3. Effectiveness varies widely for various species of microorganisms but is greatest against vegetative species.

One of the simplest and most effective decontamination methods involves the vaporization of solid paraformaldehyde within the cabinet (148,149). The disadvantages of this technique are that decontamination time may vary from 30 minutes to ten hours depending on the sensitivity of the microorganism, that the formaldehyde vapor polymerizes and condenses on surfaces depositing a film which must be removed by aeration before use, and that the deposition of this film must be excluded from electrical apparatus in the cabinet (motors, outlets) by designing suitable vapor proof barriers.

II. DESIGN OF A BIOLOGICAL SAFETY CABINET FOR A CONTINUOUS CENTRIFUGE

Respiratory tract exposure requires the greatest attention in any consideration of the design of a primary biological barrier. Some microorganisms normally considered nonpathogenic if accidentally ingested or inoculated may be virulent when inhaled in large numbers producing serious respiratory illness.

Frequently during the course of investigation of microbial products, enzymes, or cell components large quantities of cell suspensions must be processed by centrifugation. In an increasing number of studies, large volumes of microbial cultures are being processed when little is known about the potential risk to the investigator from inhalation of these organisms.

With this concern in mind, the Graduate School of the University of Wisconsin-Madison has undertaken construction of a biological safety cabinet for a continuous flow centrifuge. The purpose of this facility is specifically to protest the operator of the centrifuge from the risk of massive biological aerosols known to be generated by continuous flow laboratory centrifuges (150).

The following is a description of a class III glove box type cabinet for a continuous flow centrifuge fabricated at the University of Wisconsin Physical Sciences Laboratory (Stoughton, Wisconsin) designed in cooperation with the School of Pharmacy (as a Graduate Student Project) for the Graduate School of the University of Wisconsin-Madison.

A. Previous Designs

Several designs for class III systems for containment of aerosols produced from centrifugation of bacteria, fungi, and virus have appeared in the literature(150,151,152,138,140). The basic principles

Wisconsin Mobile Enclosed Continuous Centrifuge (WMECC) were drawn from those of Evans et. al. (150,152) used at the Microbial Research Establishment, Porton, United Kingdom. The Porton design encloses an electrically driven Sharples Laboratory Super Centrifuge (Model 1A) in a mobile welded aluminum cabinet for processing of 20 liter volumes of microbial culture. However, the Porton design has several disadvantages which have been overcome in the WMECC.

1. The Porton design does not provide for containment of the supernatant from the centrifuge in the same cabinet.
2. Decontamination of the Porton cabinet requires cumbersome removal of the electric motor from the centrifuge and storage in a protective container to avoid damage by decontaminating glutaraldehyde vapors.
3. The cabinet utilizes only glass fiber filters for biological containment of the aerosol generated by the centrifuge.
4. The Porton design does not provide a recepticle for holding spilled culture fluid should the tubing from the culture reservoir rupture or a leak develop in the centrifuge during operation.
5. The Porton design does not provide adequate work space inside of the cabinet for primary processing of the collected cell paste by methods that may also produce hazardous aerosols (such as cell disruption in a Waring Blendor, etc.).

B. Description of the Wisconsin Mobile Enclosed Continuous Centrifuge (WMECC)

1. Basic Design

Figures 57, 58, and 59 illustrate the basic components of the cabinet. The large arrows denote the path of ventilation flow. In this cabinet the air is drawn through two chambers, passing through HEPA filters (as it both enters and leaves). The cabinet is operated at

FIGURE 57

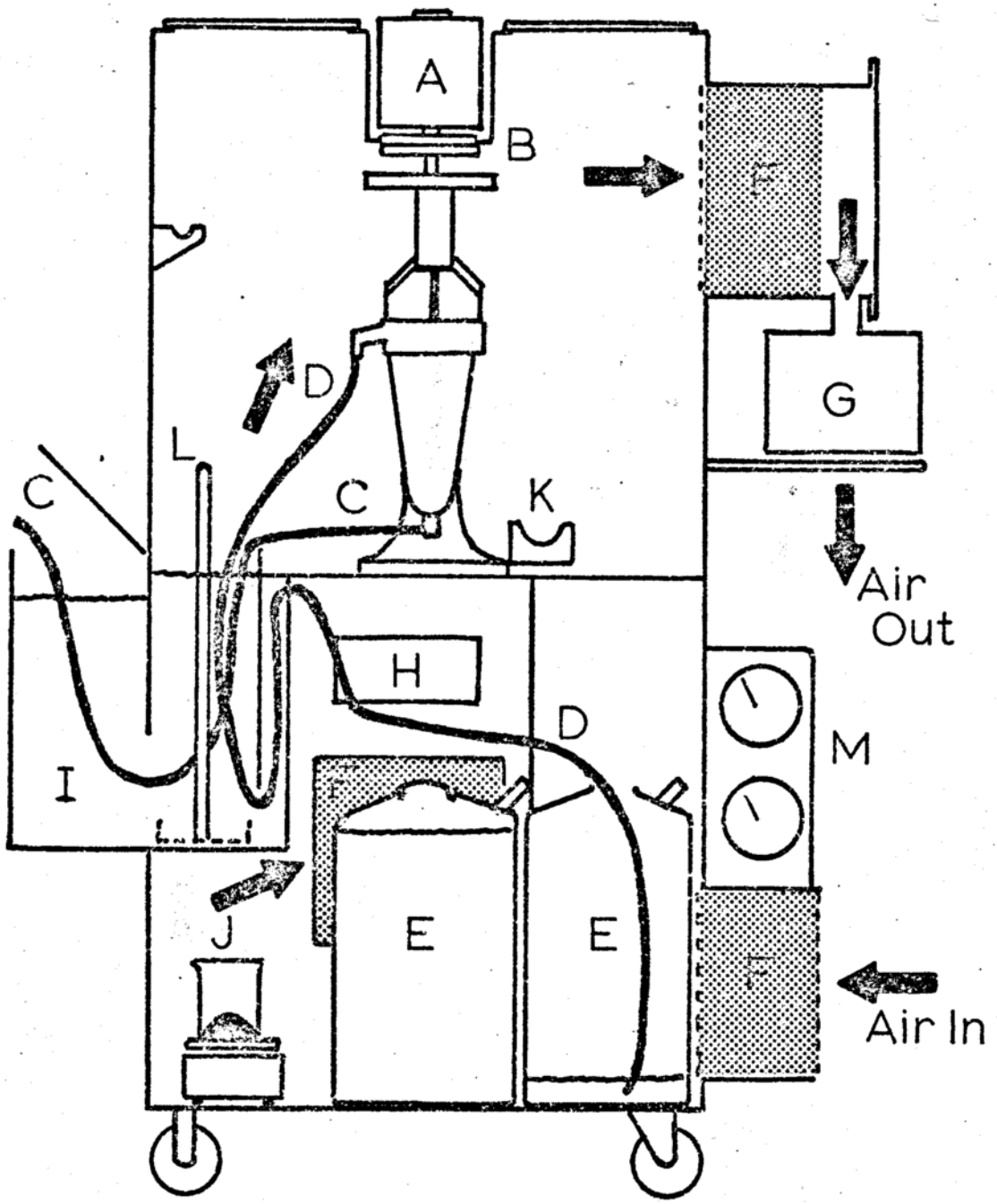


FIGURE 58

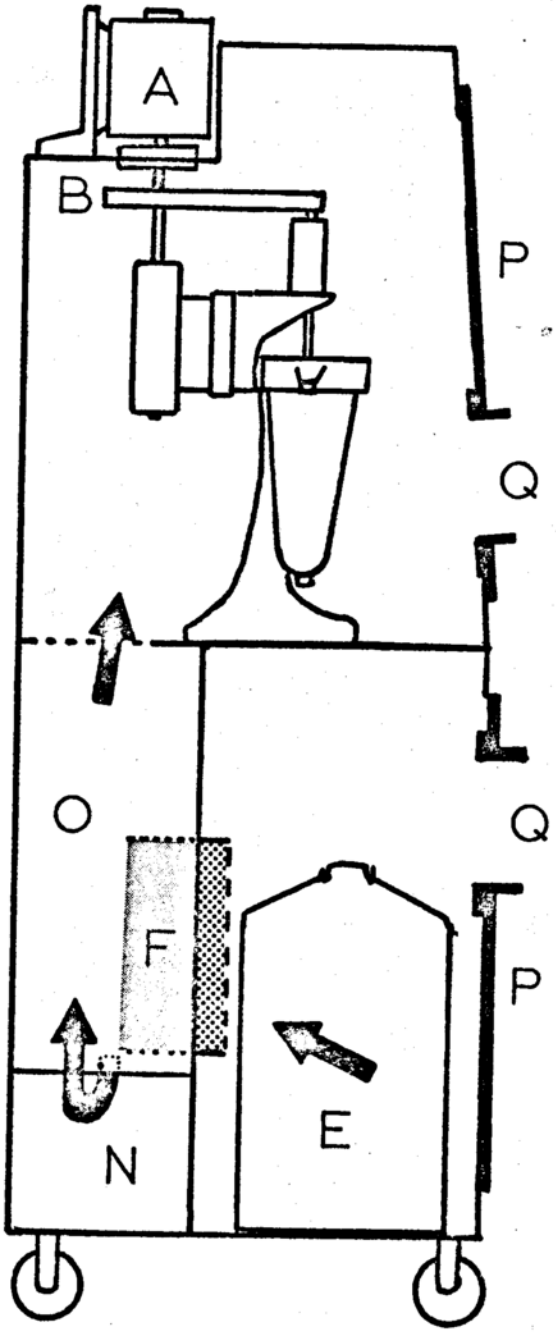
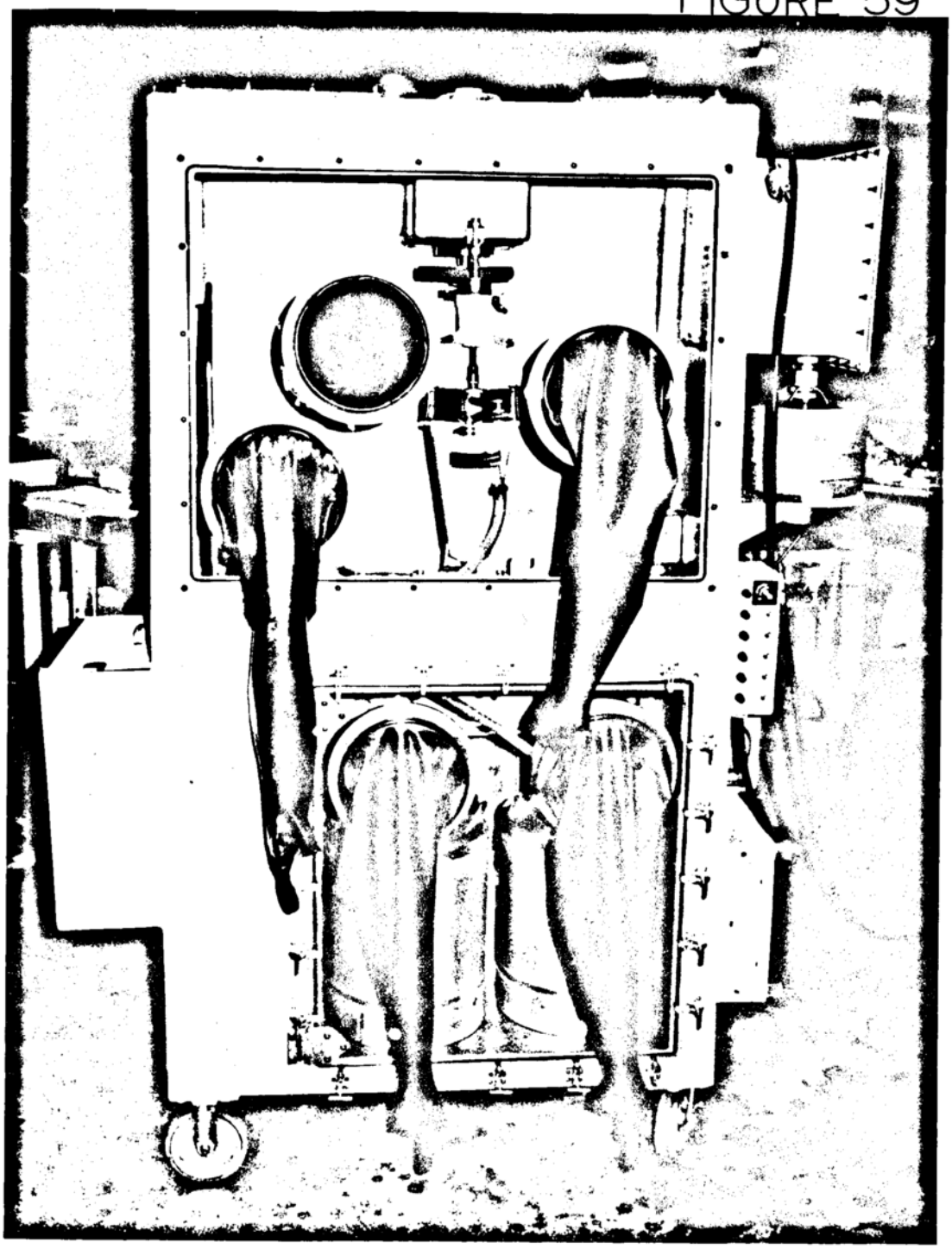


FIGURE 59



In this cabinet the air is drawn through two chambers, passing through HEPA filters (as it both enters and leaves). The cabinet is operated at between one and three inches of water negative pressure with respect to the environment. The cabinet is of all welded construction, free standing, and mobile. This cabinet can pass through a standard 6 foot 8 inch high door.

The operator is protected by working entirely through 3-8 inch rubber glove ports sealed into the front plexiglas window. The manipulations in the lower compartment are also made through sealed rubber glove ports in the collection chamber door. Should a leak occur, the operator is further protected by the pressure differential between the inside of the cabinet and the environment monitored by two Magnehelic Differential Pressure Gauges (Dwyer Series 2000, 0-5 inches of water Model No. 2005) mounted on the right side of the cabinet (figure 60).

Under normal conditions a blower ("Windjammer" Model 15540, Herback and Rademan Jr. Cat. No. TM 20K070) pulls filtered air through the lower chamber, through a second HEPA filter, up an accident well, through the upper chamber and out through a third HEPA filter. The centrifuge compartment is connected to the environment through a disinfectant liquid lock. The in-coming tubing carrying the microbial culture passes through this lock into the upper chamber. The culture can be transported to the centrifuge by gravity, pressure, or pumped. After passage through the bowl, the supernatant leaves the centrifuge through a silicone rubber tube (Silastic^{R} Dow Corning) which passes back into the disinfectant dunk tank and down into the lower collection chamber. Two seven gallom, 12 inch by 22 7/8 inch, portable stainless steel collection canisters (Alloy Products Inc., Waukesha, Wisconsin Model 73) are contained in the lower chamber (figure 61). When all of the supernatant has been collected in the canisters, the tubing from the centrifuge to the canister is discon-

FIGURE 60

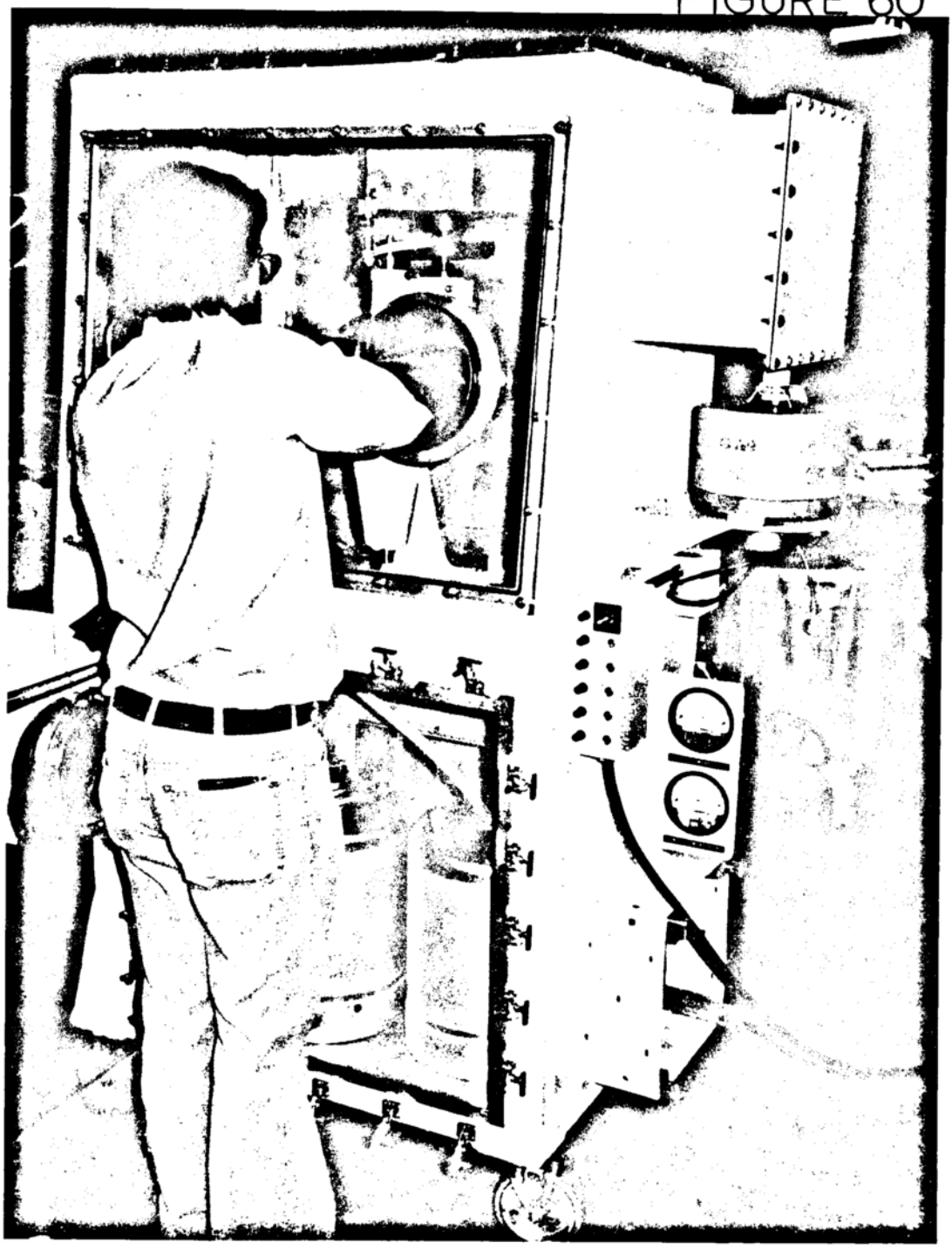
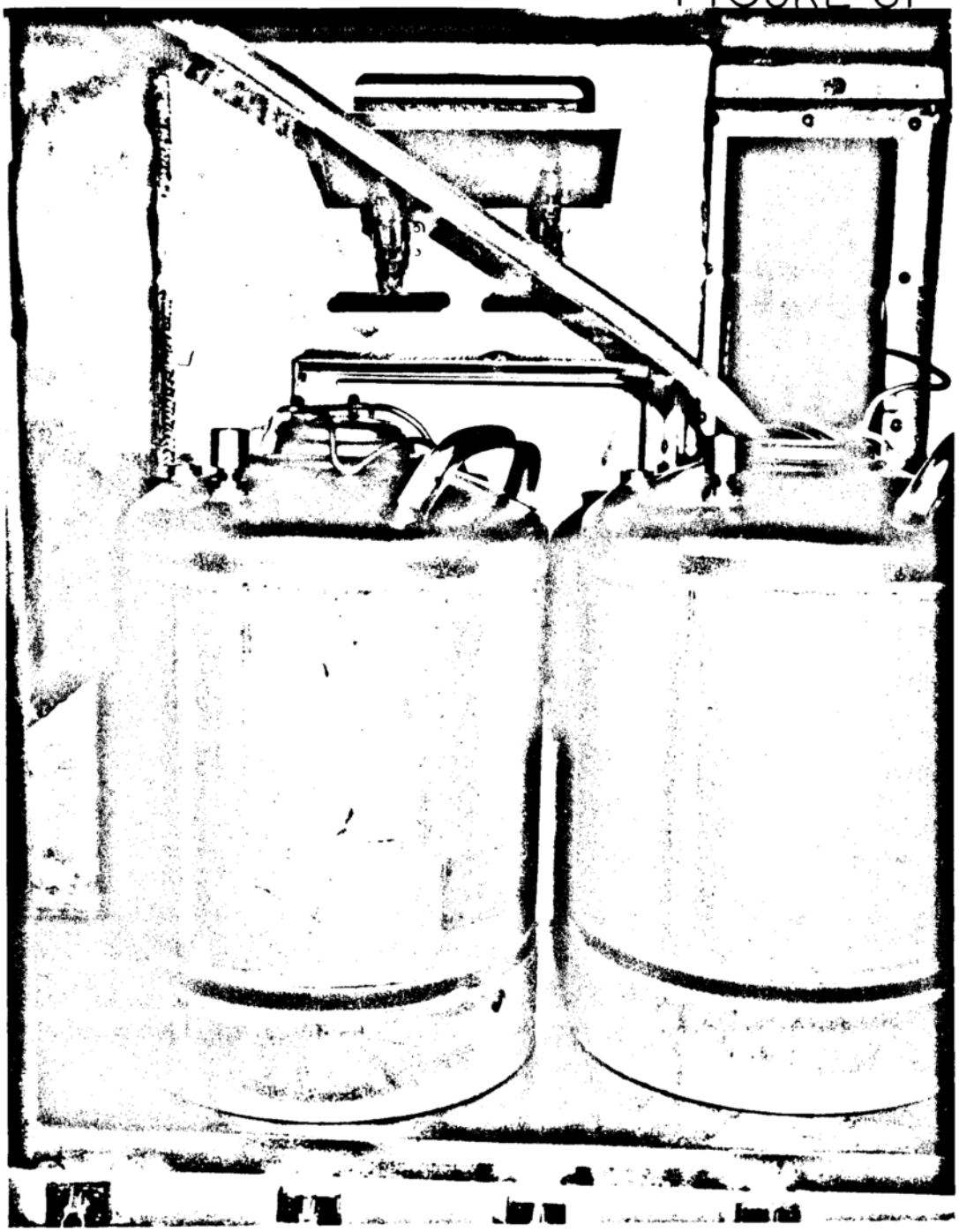


FIGURE 61



ster can be easily sealed by the operator using the rubber glove ports through the exterior door of the collection chamber (figure 62).

The bowl of the centrifuge can be removed from the centrifuge and emptied of cell paste (figure 63). A cradle for the centrifuge bowl has been installed in the centrifuge chamber to facilitate removal of the end of the bowl (by a spanner wrench) through the glove ports (figure 64). After cleaning,the bowl, cell paste, and any tools can be placed in appropriate sealable containers or sealed in autoclavable plastic bags (Royal Scientific Autoclavable Disposable Bags) and dropped into the dunk tank lock. After a determined period of time these sealed canisters can be removed from the disinfectant from the outside and autoclaved.

The sealed removable collection canisters containing the supernatant and contaminated tubing can only be removed and autoclaved after the cabinet interior has been completely sterilized.

2. Materials of Construction

The entire gas tight cabinet was constructed of 1/4 inch aluminum plate welded to an extruded aluminum frame. The leak rate of the cabinet with the filter openings blocked, five inches of water pressure, is approximately 0.014×10^{-5} cc/minute.

All windows were fabricated of 3/4 inch plexiglas (Rohm and Haas) with Viton A "O" ring seals. The bolts securing the windows were sealed to the cabinet by means of special ethylene propylene washers and acorn nuts (Type E 529-65 8,10, 1/4 inch washers, Seals and Engineering Co., Rockford, Illinois). The Viton A "O" rings and ethylene propylene washers were specifically selected for their resistance to formaldehyde vapors. The cabinet contains five windows, two located on the top (figure 63), the front centrifuge chamber window, the bottom collection chamber door, and a rear window in the lower chamber (figure

FIGURE 62

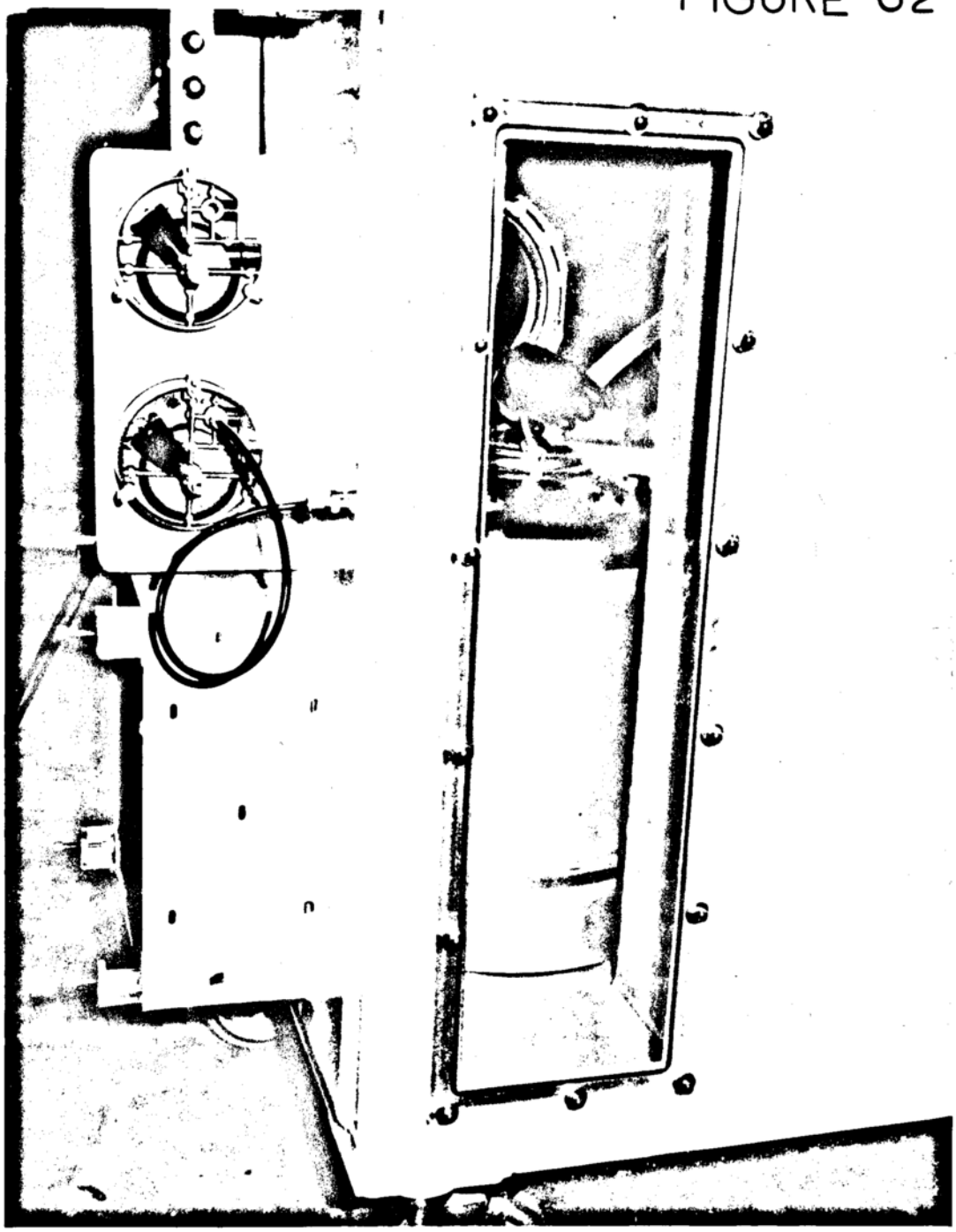


FIGURE 63

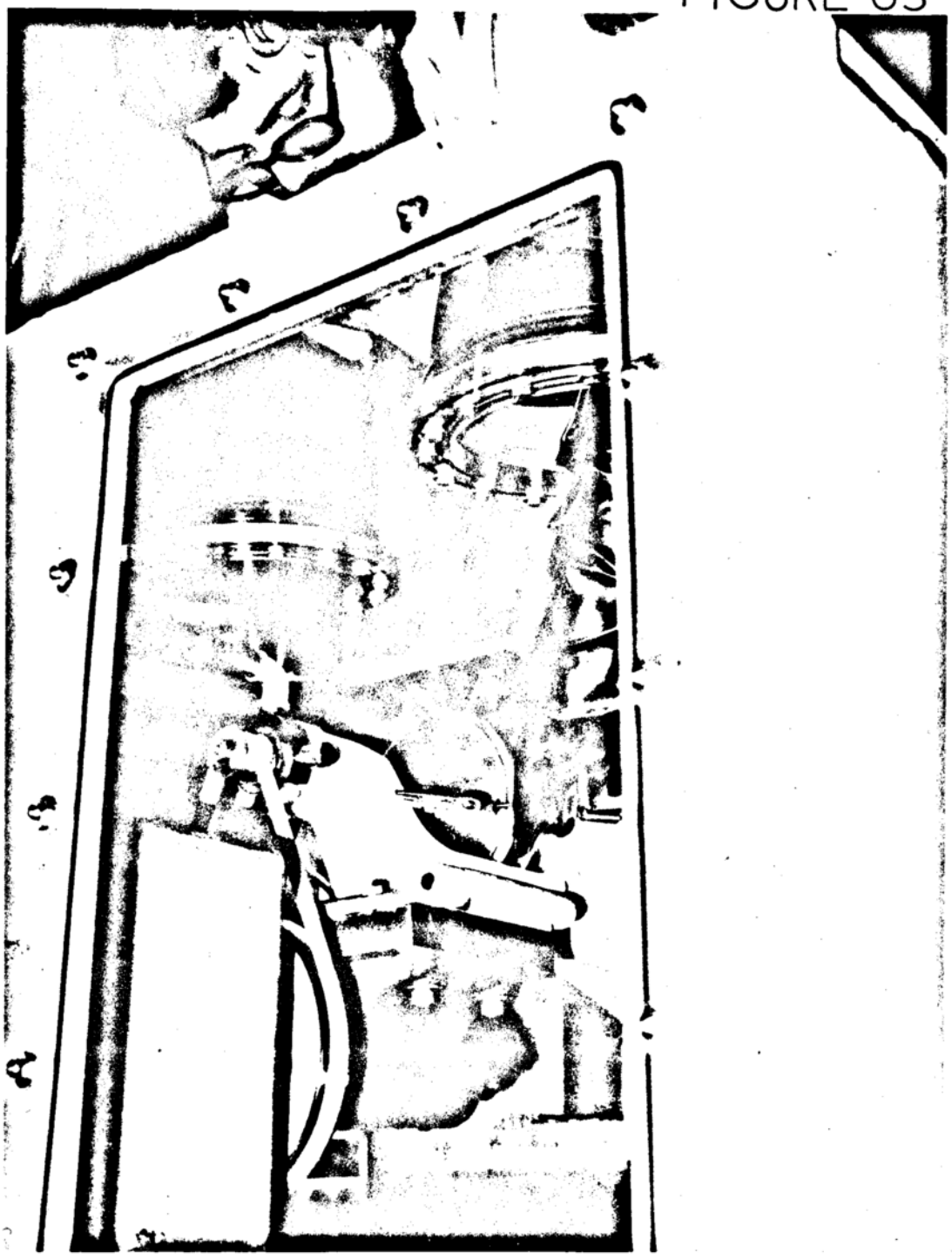
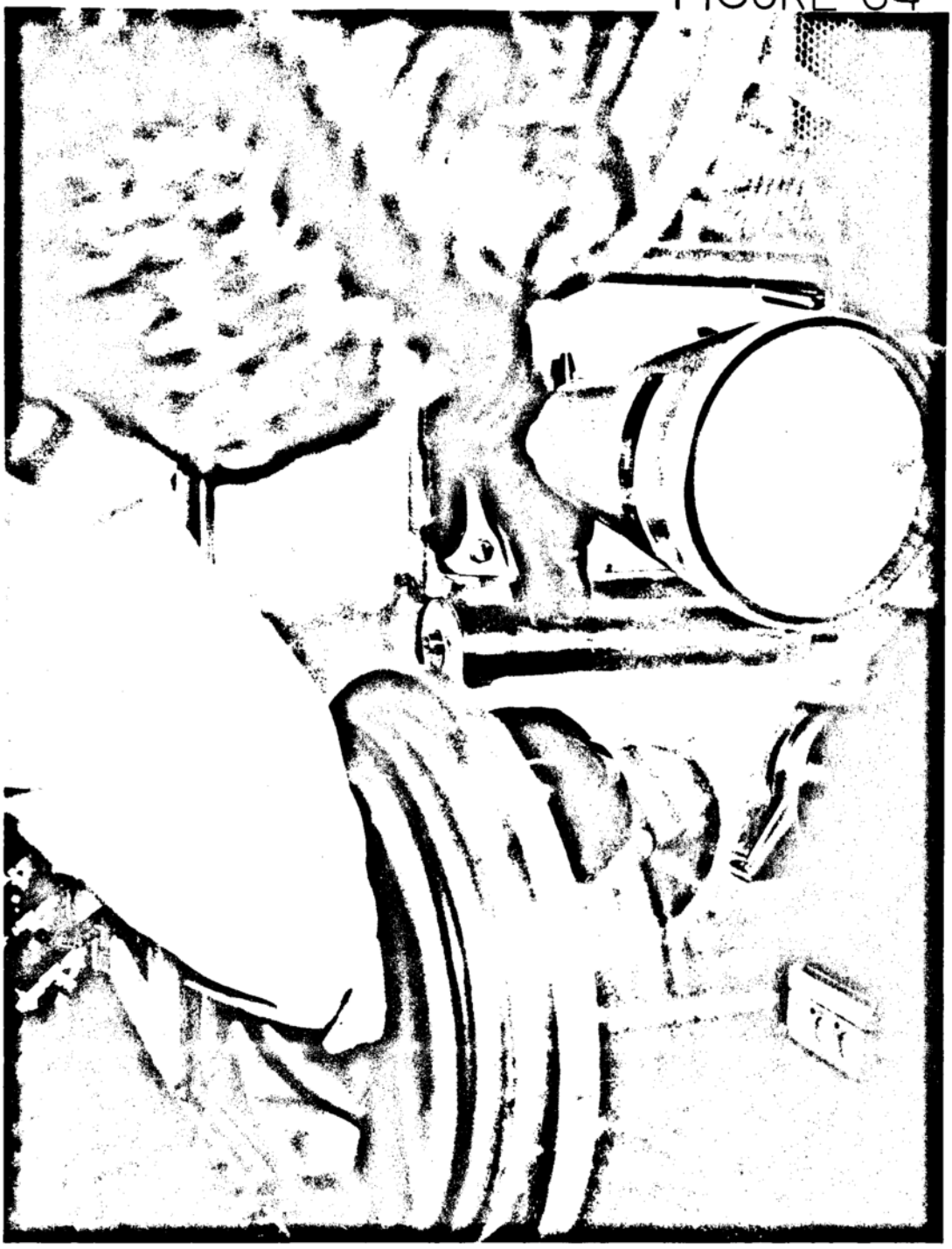


FIGURE 64



62). This fenestration allows adequate illumination by room lighting eliminating the necessity of expensive sealed lighting fixtures inside of the cabinet.

All electrical service enters the cabinet through one hermetically sealed multiple connector. Lighted switches for the centrifuge, blower, and four outlets were mounted in one box on the outside of the right side of the cabinet (figure 60). Two switched outlets are supplied in both the upper and lower chambers. The outlet boxes and conduit are metal with formaldehyde resistant outlets.

The lower compartment door is sealed by means of a Viton A "O" ring and 20 toggle clamps (De-Sta-Co Model 201-T). The blower is coupled to the exhaust filter housing by means of a teflon tube. The filter by-pass door was also sealed with a Viton A "O" ring and two toggle clamps (De-Sta-Co Model 207-TW) (figure 61).

3. Centrifuge

A CEPA Model LE continuous Centrifuge (New Brunswick Scientific Co.) is mounted in the upper chamber (figure 65). The centrifuge is driven by a belt drive mechanism using a variable speed motor (115 v, 60 Hz.) that was originally mounted on the back of the centrifuge. However, in order that the motor would not be damaged by decontamination of the cabinet with formaldehyde vapor, a magnetic drive was designed. The motor was placed outside of the cabinet, recessed in the top (figure 66), and the centrifuge was driven by a magnetic coupling through a sealed plexiglas window. The magnets for the coupling were specially machined Indow 5, 8 pole permanent magnets magnetized parallel to the thickness diameter (Permag Central cat. figure F5917, Elk Grove Village, Illinois). This magnetic drive did not compromise the 40,000 rpm maximum speed of the centrifuge.

FIGURE 65

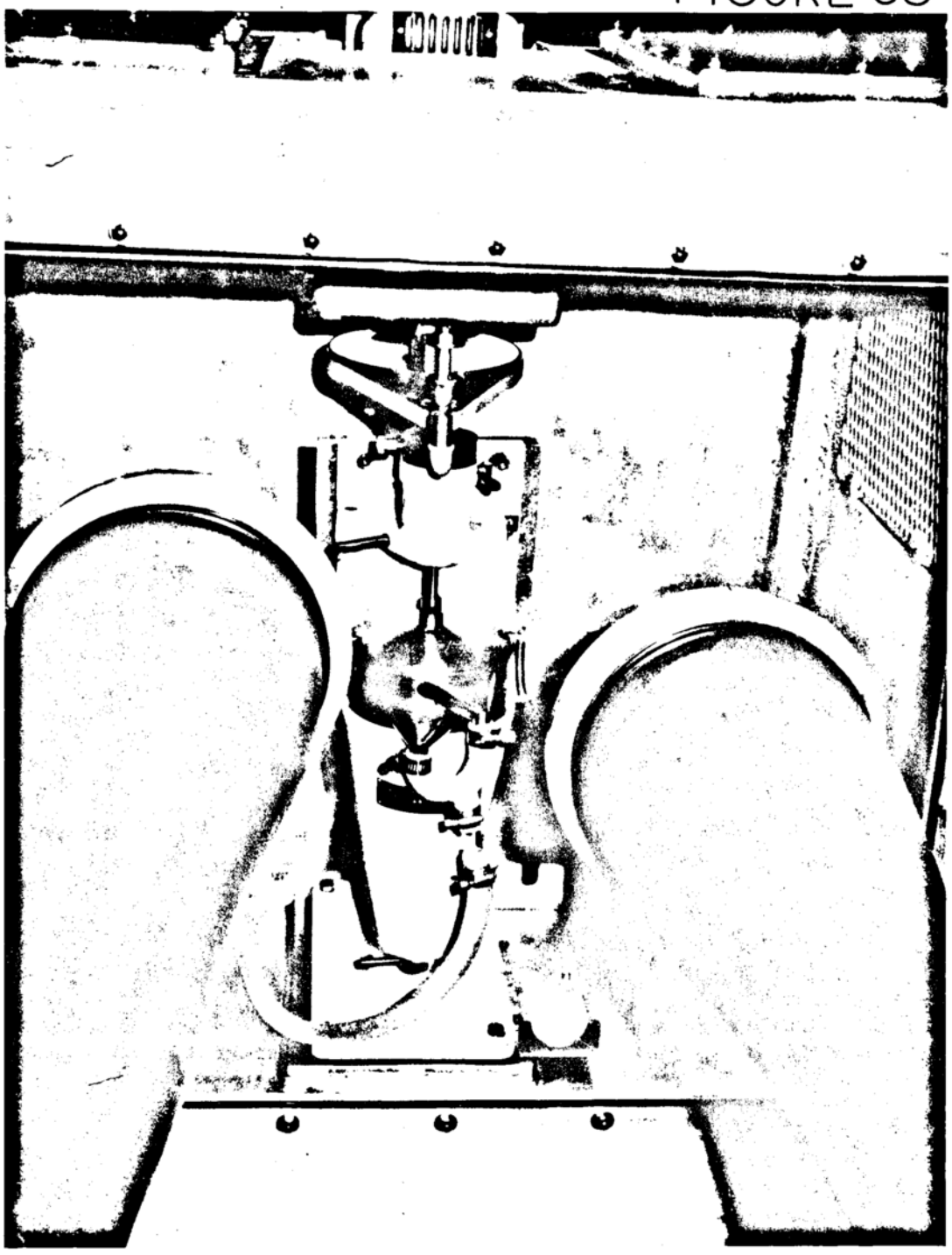
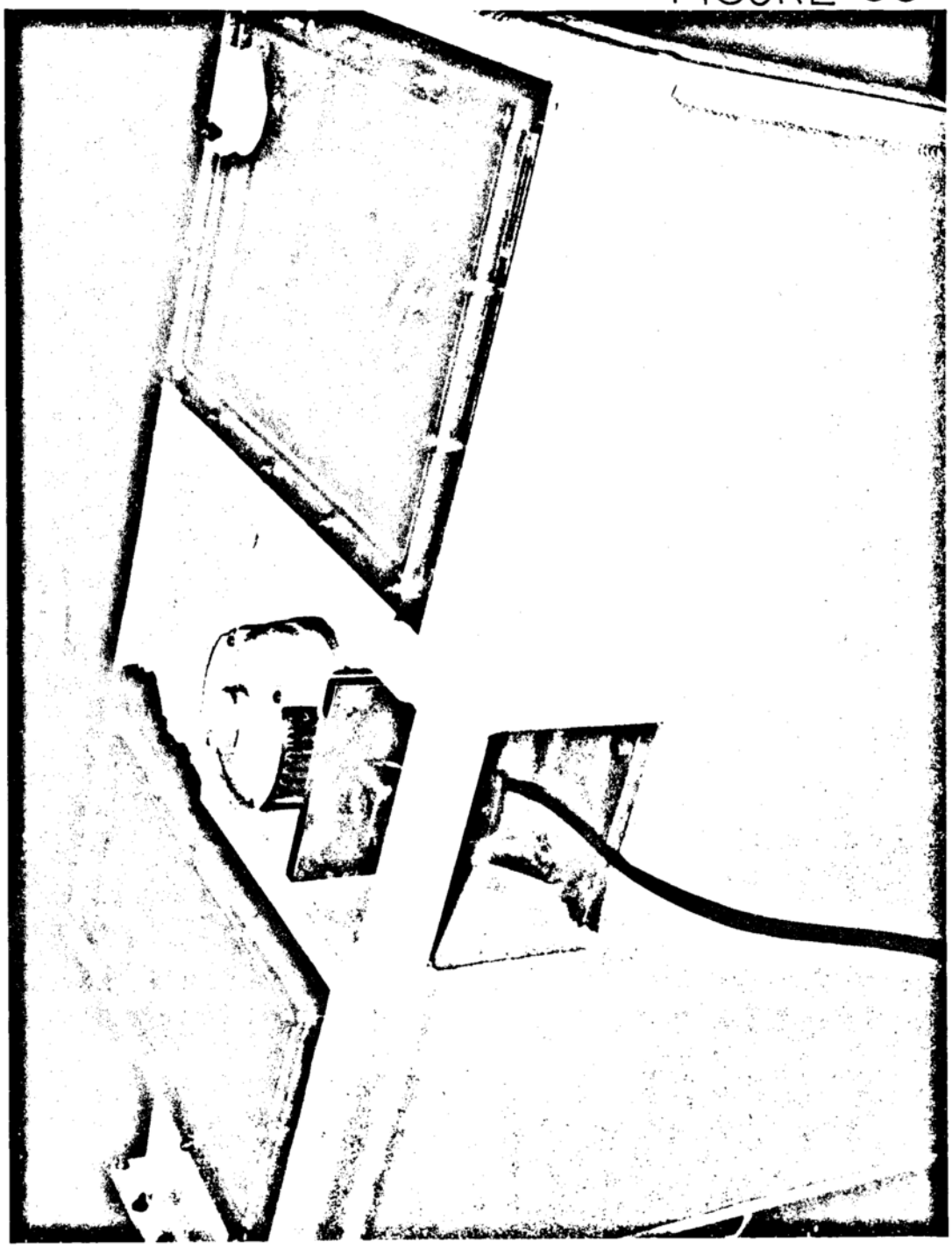


FIGURE 66



The centrifuge is equipped with an XLE-SK Serum Clarifying Bowl (316 ss) with a bottom check valve to prevent escape of fluid from the cylinder when not in motion. The 190 mm (L.) by 44 mm (I.D.) bowl is capable of processing 10-50 liters of culture per hour retaining up to 250 ml. of cell paste.

4. Filters and Disinfectant Lock

The cabinet is supplied with three 12 inch by 12 inch by 6 inch deep HEPA absolute filters (Flanders Filters, Inc. Model 7075L, size GCF) with 40 percent prefilters (Flanders Filters, Inc.). These filters are rated at a minimum filtration efficiency of 99.97 percent on removal of 0.3 micron size particles by the DOP test method. The prefilters are removable and disposable. The HEPA filters can be decontaminated in place or removed in a sealed bag for disposal.

The liquid dunk tank lock is filled with formalin or other suitable liquid disinfectant. To facilitate removal of materials from the bottom of the tank from the inside of the cabinet, a manually operated lift-basket was installed in the dunk tank (figure 57).

5. Decontamination of the Cabinet

The interior of the cabinet can be decontaminated by vaporization of solid paraformaldehyde. (148,149) Decontamination follows the method suggested by the National Institutes of Health for formaldehyde decontamination of laminar flow biological safety cabinets (149).

Two hot plates are located in the bottom chamber. On one hot plate is a beaker containing water. On top of the second hot plate is a beaker containing 9.2 grams of flake paraformaldehyde. (0.3 grams per cubic foot of volume. 30.6 cubic feet x 0.3 grams = 9.2 grams) Vaporization of this amount of paraformaldehyde will provide an equivalent concentration of 0.8 percent by weight or 10,000 parts per

million by volume of air. Formaldehyde gas is flammable in air at concentrations between seven to 73 percent. However vaporization of 0.2 to 0.3 grams per cubic foot does not approach this range. Use of excess paraformaldehyde can cause the formaldehyde to polymerize on surfaces as a white powder which can be removed by ventilation of the cabinet.

The interior of the centrifuge is first disinfected by pumping formalin through it and into the collection canister at the end of centrifugation. The blower is then shut off, the hot plate in the lower chamber with the beaker of water switched on, and the relative humidity monitored in the upper chamber. During this process the by-pass door (shown open in figure 61) separating the upper and lower chambers is opened to allow for vapors from the lower chamber to by-pass the filter separating the lower chamber and the accident well. When the relative humidity in the upper chamber has reached approximately 60 percent and the temperature is between 24°C and 30°C, the second hot plate, set at 232°C, is switched on and approximately one-half of the paraformaldehyde allowed to depolymerize. When half of the paraformaldehyde has depolymerized the blower is turned on for three to five seconds to desseminate the gas to inaccesible areas. Following complete vaporization of the paraformaldehyde the blower is again turned on for three to five seconds for thorough dessemination and the cabinet allowed to stand for a minimum of one hour. After this exposure time, and when the cabinet has cooled, the blower is switched on and the cabinet ventilated overnight to remove the adsorbed surface film of the polymer.

After this entire procedure has been completed, the collection chamber door can be opened (lower chamber door shown in figure 60) and the sealed collection canisters removed for autoclaving.

III. REQUIREMENTS FOR CERTIFICATION OF THE CABINET AS A BIOLOGICAL BARRIER

Biological testing of the centrifuge cabinet is necessary for verification of the design concepts and development of effective operational techniques and procedures.

Five types of procedures are commonly employed for surveillance and testing in microbial containment safety hoods.

Leakage testing - Testing with chlorofluohydrocarbons (Freon^{R}) should be used to validate the microbial tightness of any class III or absolute barrier system. Inability to leak molecules of freon gas is equated with the inability of microbes to enter or escape from the barrier. Leakage testing is performed under conditions of positive cabinet pressure (with air filters blocked). Usually two to six inches of water pressure is applied to the cabinet. Escaping gas is detected by a sensitive specific halogen gas detector.

Testing of filters - In most instances, microbial testing with aerosols of tracer organisms (Serratia marcescens, S. indica) will be appropriate. Microbial testing must be done under conditions of temperature and humidity that will insure the viability of airbourne microbial aerosols. The microbial aerosols are generated in order to create particles in the 0.5 to 1 micron range. Sampling is accomplished using slit or surface samplers (144,153).

Physical and chemical tests - HEPA filters may be evaluated by use of dioctylphthalate (DOP) particles with a suitable electric detector system (154). When liquid or gaseous decontaminants are used these should be particularly assayed to assure proper chemical concentration in all parts of the cabinet.

Surface contamination sampling - Moist cotton swabs or petri plates

are usually used to detect surface contamination. Results should be expressed as microorganisms per unit of surface area.

Air sampling (144)-According to the the sampling devices used, accessment can be based on microorganisms per unit volume of air. Agar settling plates can provide data on viable particles falling on a unit surface per unit of time. Slit samplers and particle impringers can provide data on the number of viable airbourne particles and particle size.

Following analysis of testing results, using acceptable testing procedures from the literature with proper statistical analysis of microbial data, the results could be submitted for federal certification to the appropriate supervisory agency. Information can be obtained from the Office of Biosafety of the Center for Disease Control, 1600 Clifton Road, N.E., Atlanta, Georgia 30333.

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